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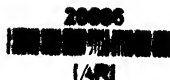


# STAIN TECHNOLOGY

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# STAIN TECHNOLOGY

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## A MYELIN STAINING METHOD FOR OLD MATERIAL

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It is sometimes necessary to study old formalin-fixed specimens of the central nervous system, or more rarely of formalin-fixed material which has been preserved in glycerin or alcohol. In these instances it may be desired to make use of a myelin stain. Specimens of this nature very often give unsatisfactory results with the usual myelin methods because the fresh material has not been fixed in the manner required by the majority of such methods.

The following method has been found useful in such cases and might also be applied to those samples of neural tissue which are found to be refractory to the usual myelin stains; it is also suitable for use with paraffin embedded tissue. A further advantage, when it is used with routine material, is rapidity, since the mordant is not applied to the specimen in bulk, and when applied to the individual section the time required may be reduced to one or two hours. It has been used in a formalin-fixed human brain stem which had been mounted in glycerin for four years as a museum specimen, and also in old 10% formalin-fixed specimens, types which gave poor results by a variety of other myelin methods.

The mordant used is iron alum which is followed by a hematoxylin stain in accordance with the usual practice of myelin staining. In the method here described the stain is used in a dilute medium of hot  $\text{Li}_2\text{CO}_3$ , intensified in cold  $\text{Li}_2\text{CO}_3$  solution, and the differentiation is completed by the use of a mixture of dilute  $\text{KMnO}_4$  and alcohol.

### METHOD

The hematoxylin stain is made up of one part of 10% ripe hematoxylin and nine parts of a solution of 5 ml. of saturated aqueous  $\text{Li}_2\text{CO}_3$  in 95 ml. distilled water. This stain must be used at once. The block, after preliminary washing and dehydration with dioxane, is embedded in paraffin, cut at 20 to 25  $\mu$  and mounted in glycerin-albumin. Then it is treated as follows:

1. Bring the sections down to water and mordant overnight in 4% iron alum (Heidenhain).

2. Following the mordant, wash the section quickly in distilled water.

3. Place the slide on a hot plate at 50° C. and cover with a few drops of the stain for 5 minutes.

4. Flood the section with fresh stain and leave for 10 to 15 minutes. It may be noted that the heating of the section, apart from the known acceleration in staining, brings about a fairly satisfactory differentiation.

5. Remove the slide from the hot plate, drain, and wash off the excess stain completely with the dilute  $\text{Li}_2\text{CO}_3$  solution.

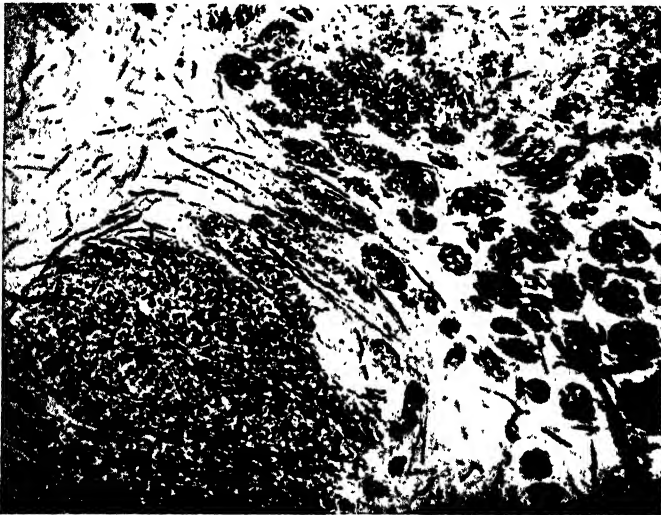


Fig. 1. Fine fiber tracts at the lateral side of the floor of the IV ventricle (fasciculus solitarius and area fasciculata). Human brain stem from museum specimen four years old.  $\times 70$ .

6. Wash out the  $\text{Li}_2\text{CO}_3$  with distilled water.

7. Flood the section with 90% alcohol for 2 to 3 minutes.

8. Take one part of a 0.25% solution of  $\text{KMnO}_4$  in distilled water and add it to nine parts of absolute alcohol; a few drops of this mixture are at once placed on the slide and renewed two or three times until the gray matter can be seen to assume a light yellow color. This should take 1 to 3 minutes.

9. The section is then washed in 90% alcohol, dehydrated in absolute alcohol, cleared in xylene and mounted in balsam.

The myelin fibers appear bluish black against a pale yellow background and the transparent cell masses are lightly toned or almost colorless. If the differentiation is carried out carefully, the finer fibers show up in good contrast, as may be seen from the accompanying photomicrograph of a section through the fasciculus solitarius and neighboring structures. The best results are obtained with brain stem and spinal cord. Some thick sections tend to become detached, due it appears to the action of the  $\text{Li}_2\text{CO}_3$  rather than to the warming of the slide; since the staining is carried out on the slide, the sections can be manipulated without much difficulty.

My thanks are due to Mr. Albert Young for the photomicrograph.



## HISTOCHEMICAL TESTS FOR PROTEINS AND AMINO ACIDS; THE CHARACTERIZATION OF BASIC PROTEINS

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**ABSTRACT.**—For cytophysiological work it is important to have ways of demonstrating proteins and amino acids and especially of characterizing basic and non-basic proteins. The author presents a review of the more usually employed histochemical reactions for amino acids and proteic compounds in general, with several modifications which increase their sensitivity, or specificity and localization. The author describes the histochemical arginine reaction, recently introduced by him, by means of which the characterization of basic and non-basic proteins can be easily accomplished in every laboratory without costly apparatus; this reaction serves also for the demonstration of proteins in general. The application of protein histochemical tests for quantitative purposes is discussed in connection with the characterization of the basic proteins and the determination of the relative concentration and the active metabolic changes of proteic compounds.

### INTRODUCTION

The histochemical characterization of proteins and their components has recently acquired a renewed importance in connection with cytophysiological work on nucleoproteins and other cell components (see, for instance, Brachet, 1940; Caspersson, 1940, 1941; Hydn, 1943; Serra and Queiroz Lopes, 1944, 1945; and the bibliographies in Schultz, 1943 and Greenstein, 1944). For these studies it has become of importance not only to demonstrate the proteic nature of a certain cell structure, but also to distinguish between different protein classes and especially between basic and non-basic proteins.

One of the means of accomplishing this characterization is ultra-violet microspectroscopy, developed by Caspersson and co-workers (detailed descriptions in Caspersson, 1939, 1940). A strong absorption at 2600 Å indicates the presence of purine and pyrimidine compounds, which generally belong to nucleotides, of the pentose (ribonucleic) or the desoxypentose (thymonucleic) types. Owing to the presence of tyrosine, tryptophane and phenylalanine, the protein component of the nucleoproteins gives an absorption at about 2750–2900 Å; according to Caspersson, a shift of the absorption maximum

in this region to the longer wavelengths signifies that the protein is rich in hexone bases, which is characteristic of the basic or histone type proteins. If such a shift does not occur, the protein is considered to be of a non-basic or 'superior' type.

However, the demonstration of the basic type of the nucleoproteins by this method has been doubted, because histones can give an absorption at 2800 Å, the lower wavelengths of the absorption region (Mirsky and Polister, 1943; cited by Schultz, 1943). It is therefore of interest to have another independent means of identifying the basic proteins. We present here a description of such a method, which is practicable in any laboratory without costly apparatus; at the same time we make, so far as is possible for us, an up-to-date critical review of the histochemical tests for proteins and amino acids.

#### PREPARATION OF THE MATERIALS

The materials may be sectioned by the freezing microtome, the reactions being executed without fixation, but in general for histochemical work it is better to fix the pieces in convenient fixatives. For all histochemical tests described below, except the reaction for SH groups, we have used with good results a fixative composed of 2 vol. of 96% alcohol, 1 vol. of commercial formalin (40% formaldehyde) and some drops of glacial acetic acid in 10 ml. of the mixture. Afterwards the pieces are well washed with running, and finally with distilled, water.

Of course, other cytological fixatives may be also employed but it is necessary to avoid colorations or precipitates caused in the tissues by the fixatives. Moreover, the materials may be embedded in paraffin and sectioned in the usual manner or stored for some time in 70% alcohol, but in these cases it is better to harden the tissues again before the reactions are executed.

This hardening, which is also necessary when fixatives without formaldehyde have been used, is done by a treatment with 10% formaldehyde (1 vol. of commercial formalin to 3 vol. of water) for 12-24 hours, followed by a thorough washing. Other references to the preparation of the materials will be made when dealing with each individual reaction.

#### TESTS FOR AMINO ACIDS AND THE LOWER PEPTIDES

When operating on fixed and washed material, in general it is not expected to find free amino acids, owing to the fact that these are relatively soluble. The majority of these compounds dissolve in water, though some of them, like aspartic acid, cystine, leucine,

methionine, phenylalanine, tyrosine and tryptophane are difficult to dissolve in cold water. Only in certain materials, such as plant sproutings and cotyledons, there is a relatively great amount of free amino acids; on the contrary, what is generally of interest is the characterization of amino acids bound in proteins.

In this section descriptions are given of the two histochemical reactions more commonly used to-day for the characterization of free or bound amino acids in general, and another test especially employed for sulfhydryl groups. Other reactions specific for certain amino acids will be dealt with under "Tests for proteins".

1. *The ninhydrin reaction* (Klein, 1928; Winterstein, 1933; Lison, 1936; Lloyd and Shore, 1938; Ries, 1938). Procedure of Serra and Queiroz Lopes, 1945: The fresh materials or the fixed pieces or sections are immersed in a mixture of equal volumes of (a) a 0.4% solution of triketo-hydrinden-hydrate (ninhydrin) in distilled water and (b) a phosphate buffer of pH 6.98. The reaction vessel, for instance a small watch glass covered with a glass plate, is now placed on a boiling water bath, where it is allowed to stand in the vapor for 1-2 minutes after it has reached boiling-point. A blue or violet coloration, which develops while on the bath or after cooling, indicates the presence of amino acids, free or bound in peptides or proteins.

For microscopical observation the sections or pieces are mounted directly in pure glycerin, and the tissues squeezed, if necessary. The preparations, which can be cemented with a cement made by adding 80 g. of colophonium to 20 g. of carefully heated lanolin (Romeis, 1932) must be observed in the same day, since the coloration rapidly fades away.

The ninhydrin solution must be freshly prepared, as it is unstable, and the phosphate buffer must not be too concentrated; the mixture of 6 ml. of a *M*/15 solution of secondary sodium phosphate (11.1876 g.  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  per liter) and 4 ml. of *M*/15 primary potassium phosphate (9.078 g.  $\text{K H}_2\text{PO}_4$  per liter) gives a pH of 6.98 and has always given us good results.

With regard to *specificity*, it is known that the reaction is given by all amino acids except proline and hydroxyproline, by peptides, proteins, and also by a series of other compounds like amines, aldehydes, sugars with free aldehyde or keto groups and by ammonia and ammonium salts. However, with compounds other than amino acids and proteides, the reaction is much less sensitive and sometimes it gives a more reddish color. In general it is easy to exclude the possibility of these compounds being present, by their solubility and localization. It must also be remembered that the intensity of the

ninhydrin reaction varies according to the nature of the amino acid and the binding of this in the peptides.

The coloring formed during the reaction can diffuse and be *absorbed* by several cell structures. This commonly happens when the heating is exaggerated and when compounds easily soluble are present, for instance after a weak fixation. It is, therefore, recommended to employ fixatives which harden the tissues, as we have said above. To be sure that a secondary impregnation or adsorption of the coloring has not taken place, the following test may be executed: A small weight (some milligrams) of a pure amino acid, such as glycine, is dissolved in distilled water; an equal volume of phosphate buffer of pH 6.98 and a few drops of 0.4% ninhydrin solution are added; it is boiled slowly and cooled for 20–30 minutes. The ninhydrin employed must be completely consumed — by addition of more amino acid solution. The colored liquid of this reaction is now used to immerse the pieces, with boiling, etc., as for a ninhydrin reaction. If then a certain structure shows a coloration, this means that an absorption or adsorption has taken place and a positive ninhydrin reaction in the same structure does not necessarily demonstrate a proteic or amino acid nature.

2. *The alloxan reaction* (Winterstein, 1933; Lison, 1936; Ries, 1938). An alcoholic 1% solution of alloxan gives with amino acids and proteic compounds a pink coloration, after a long time at room temperature, or rapidly if the reaction is activated by heating in a boiling water bath. In our experience, this test is relatively insensitive; besides this, the coloring formed diffuses easily, so that the reaction can be indistinctly localized. With fixed materials the reaction is weak.

The test must be carried out in neutral solutions; this is attained by addition of a phosphate buffer, as described for the ninhydrin. This reaction is not specific for amino acids and proteins, as it is also given by compounds with free  $\text{NH}_2$  and perhaps SH groups (see Winterstein, 1933).

3. *The reaction for sulfhydryl groups* (Lison, 1936; Ries, 1938; Serra and Queiroz Lopes, 1945). This reaction has been extensively used for the study of the distribution of the tripeptide glutathione. One of the better methods of accomplishing the reaction is that of Giroud and Bulliard (see Lison, 1936), which gives a stable red coloration, while other methods produce a violet color rapidly fading away.

The pieces are immersed for some seconds (in general an excess of time does no harm) in a 5% aqueous solution of zinc acetate. Directly afterwards they are treated with a 10% aqueous solution of sodium

nitroprusside, containing about 2% concentrated ammonia. The pieces acquire a bright red coloration, which attains its maximum in 3-5 minutes. Afterwards they are mounted in pure glycerin for microscopic observation, if necessary with a preliminary washing in distilled water.

The materials may be studied freshly or after fixation. It must be noted, however, that the majority of the fixatives hinder the reaction. We obtained good results with a fixation in 10% neutral formaldehyde during 2-15 hours at room temperature. A more prolonged action of this fixative also hinders the reaction; it is recommended, therefore, that if possible 2-4 hours of fixation be used.

The results of the reaction have different meanings according to the fixation, washings, etc., because the glutathione is partly soluble. When the tissues are treated several times with a 10% solution of trichloroacetic acid for 15 minutes, the glutathione is dissolved and only "fixed", that is, proteic sulfhydryl groups remain in the preparation. It is still possible not only to demonstrate the existing SH groups but also to reduce SS groups to SH groups, by means of a pre-treatment of the materials with a solution of 10% KCN for 10 minutes in a small stoppered bottle (the cyanide solution can be weakly alkalinized with potassium hydroxide, to make its use safe).

The reaction has been recognized as well localized, but in case of doubt a test of secondary impregnation can be made in the way described above for the ninhydrin.

### TESTS FOR PROTEINS

Under this heading will be described tests which are also given by free amino acids, but whose characterization is principally important when they are bound in proteins.

1. *The biuret reaction* (Klein, 1928; Winterstein, 1933; Lloyd and Shore, 1938; Calvery, 1938). The pieces are immersed in strong NaOH or KOH solution in a watch glass and some drops of a 1% aqueous solution of  $\text{CuSO}_4$  are then added with stirring. A blue-violet coloration indicates the presence of peptides or proteins.

The reaction is given by the peptide linkage when the peptides are composed of at least three amino acids. The color is more reddish with the simpler peptides. For cytological or histological work, the reaction has the disadvantage of requiring a strong alkaline reaction, which tends to dissolve the protoplasm. To avoid a serious dissolution the tissues must be hardened, for instance with formalin (10% formaldehyde during 24 hours, followed by a thorough washing). This reaction has also the disadvantage of being insensitive.

2. *The xanthoproteic reaction* (Klein, 1928; Winterstein, 1933;

Lison, 1936; Calvery, 1938; Lloyd and Shore, 1938; Ries, 1938). The pieces are treated for some minutes with concentrated  $\text{HNO}_3$  until they become intensely yellow. After a washing in distilled water, immerse in a diluted ammonia solution, or expose the pieces to ammonia vapors. The color changes to orange. The observation can be made by mounting directly in pure glycerin.

The reaction is due to the presence of tyrosine, phenylalanine or tryptophane in the protein molecule, and is also given by all phenolic compounds. Among the peptides, only the protamines do not show a positive reaction. To withstand the treatments, a strong fixation is recommended, though the reaction can also be performed on fresh materials.

3. *The arginine reaction* (Serra, 1944). This is the application to histochemistry of the arginine reaction used in microchemistry. The procedure of Serra (1944b) is the following:

(a) Before the reaction the pieces or sections are hardened with 10% formaldehyde during 12–24 hours, the formalin being afterwards well washed out. (If the fixative contains formalin this step can be omitted).

(b) Immerse the pieces for 15 minutes in a mixture consisting of 0.5 ml. of diluted  $\alpha$ -naphthol; 0.5 ml. of  $N$  NaOH; and 0.2 ml. of 40% aqueous urea solution. The diluted  $\alpha$ -naphthol is prepared at the moment of use by diluting a stock solution (1% crystallized  $\alpha$ -naphthol in 96% alcohol) 1:10 with 40% alcohol. The watch glass containing the liquids is placed in an ice-bath and the temperature of the reaction fluid inside it must be  $0-5^\circ\text{C}$ .

(c) After 12–15 minutes add 0.2 ml. of a 2% solution of NaOBr. This reagent is allowed to act for 3 minutes and the solution must be well stirred during this time. The 2% NaOBr must be freshly prepared by pouring 2 g. (or approximately 0.7 ml.) of liquid bromine into 100 ml. of 5% NaOH, with agitation and cooling.

(d) Add another 0.2 ml. of 40% urea solution, stir, and immediately afterward,

(e) Add another 0.2 ml. of 2% NaOBr and stir well. The coloration attains its maximum after 3–5 minutes and would last only for a short time if it were not stabilized. To stabilize the coloration:

(f) Take the pieces out of the reaction mixture and immerse in pure glycerin for 2–3 minutes and then transfer to fresh glycerin. Repeat the operation another two or three times. The passage through 4 glycerin baths is sufficient to stabilize the coloration for some months, even if the pieces are left at room temperature. (We have not mentioned this improvement in any previous publication).

Besides this procedure, which we may call the normal method,

there is also another method which results in *stronger colorations* and very satisfactory preparations. To accomplish this, after step *e* the pieces are taken off the reaction liquid and immersed in NaOBr solution for not more than 3 minutes. Afterwards the coloration is stabilized in glycerin, as in the normal procedure. The pieces are mounted and observed in pure glycerin. (See Fig. 1-3.)

This reaction is specific for guanidine derivatives in which only one H-atom of one amino group is substituted by a radical of the alkyl or fatty acid type. In proteic compounds it is specific for arginine. As all proteins hitherto analyzed possess arginine in their molecules, the reaction may be used to demonstrate the presence of proteins in general, other compounds with a reactive guanidine group being rare. The test may also be used to characterize the basic proteins (see below).

4. *The tyrosine reaction (Millon's modified)*. (Klein, 1928; Winterstein, 1933; Lison, 1936; Calvery, 1938; Lloyd and Shore, 1938; Ries, 1938). The procedure of Serra and Queiroz Lopes, 1945, which gives better results than the Millon's reaction, is as follows:

(a) Immerse the objects for 30 minutes in a few milliliters of the mercuric solution (composition:  $\text{MgSO}_4$ , 7.5 g.;  $\text{MgCl}_2$ , 5.5 g.;  $\text{Na}_2\text{SO}_4$ , 7.0 g.;—dissolved in 85 ml. of distilled water to which 12.5 g. of concentrated  $\text{H}_2\text{SO}_4$  is added; after dissolving diluted to 100 ml. with distilled water). Perform the treatment in a small glass stoppered bottle, placed in a water bath which is maintained at  $60^\circ \text{C}$ .

(b) After the 30-minute treatment, cool the bottle in running water and allow to stand at room temperature for 10 minutes.

(c) Dilute the mercuric solution in the bottle, by addition of an equal volume of distilled water.

(d) Develop the color, adding now some drops of a freshly-prepared 1 *M* solution of sodium nitrite (6.9 g.  $\text{NaNO}_2$  in 100 ml. of water).

The coloration attains its maximum in 3 minutes and lasts for some months, though it fades gradually with time. The materials are mounted and observed in pure glycerin, where they can be squeezed or squashed, if necessary. (See Fig. 4-6.)

The reaction is principally due to the presence of tyrosine in the protein molecule, and is also produced by other phenolic compounds. The method here described gives with tryptophane only a transient coloration, which lasts no more than a few minutes; it is hoped, therefore, that by this procedure this histochemical test reveals only the tyrosine in the proteins.

5. *The tryptophane reaction* (Klein, 1928; Winterstein, 1933). Reaction of Voisenet-Fürth, according to the procedure of Serra and Queiroz Lopes, 1945a:

FIG. 1. Testis of a male frog. Fixation: alcohol-formol-acetic acid. Arginine reaction. Mounted in glycerin and squashed. At the lower right a group of spermatozoa heads, at the left several spermatocytes in I division of the meiosis. Photographed with a green filter.  $\times 465$ .

FIG. 2. A group of spermatozoa of the snail *Helix aspersa* Mull in an advanced stage of the spermiogenesis. Fixation: alcohol-formol-acetic acid. Arginine reaction. Mounted in glycerin. At the top their heads, below them the tail in formation and the remnants of the granulous cytoplasm.  $\times 465$ .

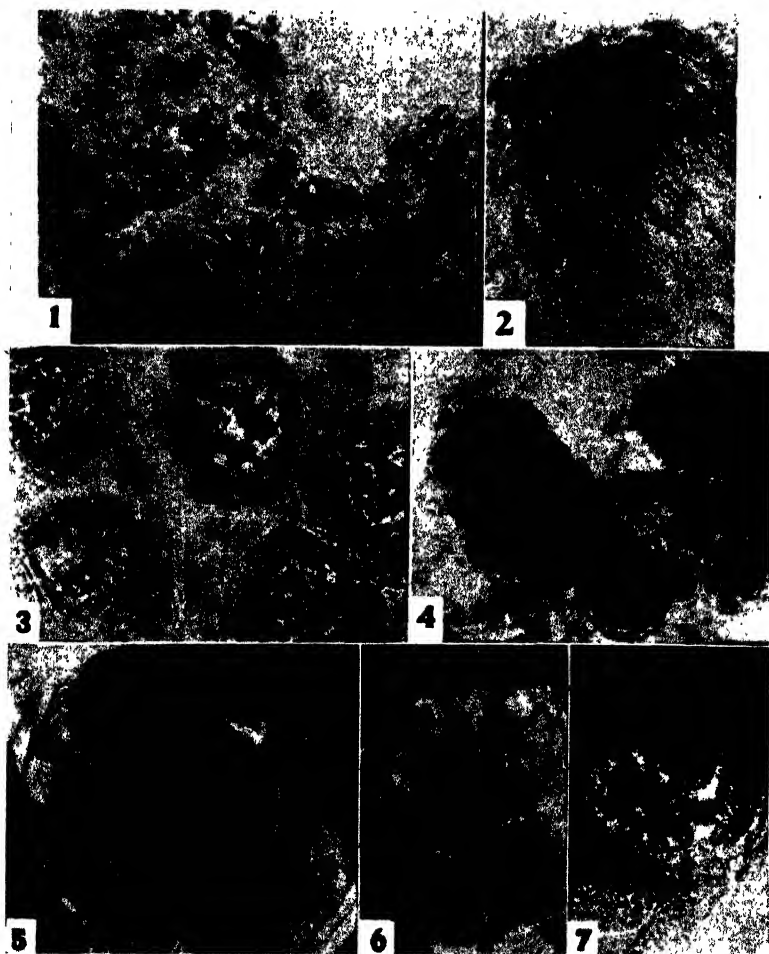
FIG. 3. Pollen mother cells of *Vicia faba* L, fixed as for Fig. 1 and 2. Arginine reaction. Squashed in glycerin. The meiotic chromosomes give a strong reaction, the cytoplasm is also relatively rich in basic proteins.  $\times 750$ .

FIG. 4. Ovary of a female frog. Fixation alcohol-formol-acetic acid. Tyrosine reaction. Mounted in glycerin. There is a great difference in the reaction given by the cytoplasm and the nucleus (while the arginine reaction is almost as strong in both, which makes difficult to photograph the ovules after an arginine coloration). Photographed without filter.  $\times 350$ .

FIG. 5. An ovule of the same frog as in Fig. 4. Tyrosine reaction; mounted in glycerin. In the nucleus a strong reaction is given by the small and numerous nucleoli, the chromosomes and perhaps also by the nucleoplasm. Photographed without filter.  $\times 465$ .

FIG. 6. A nucleus of the salivary glands of a *Chironomidae* larva. Fixation as for Fig. 4 and 5. Tyrosine reaction. Only a chromosome and the nucleolus are seen, the other three chromosomes are on other planes. Photographed without filter.  $\times 580$ .

FIG. 7. A cell of the salivary glands of a *Chironomidae* larva. Fixation: alcohol-formol-acetic acid. Mounted in glycerin. Several chromosomes and the great nucleolus are seen within the nucleus. The light violet coloration is very difficult to photograph, though it can be easily observed at the microscope. Photographed with a yellow filter.  $\times 465$ .



(a) **Harden** the fixed pieces in 10% formaldehyde for at least 1-5 hours (an unnecessary step if a fixative with formalin has been employed); then wash well.

(b) **Immerse** for 3-5 seconds in an aqueous solution of sodium silicate ( $d=1.1$ ). When the materials are sufficiently hardened this step may also be omitted; it is recommended, however, that the coloration should be tried both with and without it.

(c) **Immediately afterwards**, immerse the pieces in the Voisenet reagent for 10-15 minutes, in a small glass stoppered bottle. This reagent is composed of 10 ml. concentrated HCl to which is added, with a thorough stirring, one drop of 2% aqueous formol and one drop of 0.5% aqueous  $\text{NaNO}_2$ . The reagent is prepared freshly every day and the nitrite solution must also be freshly made.

(d) **Mount** directly in glycerin and observe, with squeezing, if necessary. As the coloration fades, it is necessary to observe the preparations on the same day.

The reaction is given by indolic compounds, and in proteins it is specific for tryptophane, which reacts even when bound. The localization of the reaction seems to be satisfactory and the *sensitivity* is sufficient for it to be used in cytophysiological work. (See Fig. 7.)

5. *The diazo reaction* (Klein, 1928; Winterstein, 1933; Lison, 1936). According to Serra, 1945, modified: Treat the pieces for 2-3 minutes with a saturated aqueous solution of sodium carbonate; afterwards add some drops of the diazo reagent and stir the liquid well. Observe in glycerin. (The coloration develops rapidly and lasts for some days.) Preparation of the diazo-reagent: into a 50 ml. flask immersed in an ice bath, pour 1.5 ml. of a sulphanilic acid solution (dissolve 0.9 g. of pure sulphanilic acid in 9 ml. of concentrated HCl and add water to 100 ml.); add 1.5 ml. of a 5% aqueous solution of  $\text{NaNO}_2$ , shaking the flask meanwhile. After 5 minutes in the ice bath add, also while shaking, another 6 ml. of nitrite. After 5 minutes fill up to 50 ml. with cooled distilled water. The reagent must be prepared every day and kept in the ice chest.

The reaction gives an orange or yellow color with the histidine and the tyrosine of the proteins. According to Klein, 1928 (see also Romeis, 1932) it would be possible to distinguish between histidine and tyrosine if a xanthoproteic reaction were previously performed upon the same pieces, followed by the diazo reaction. In these conditions only the histidine would show a red color, but the two processes seriously damage the structures, so much that the coloration may finally not be distinctly localized.

Lison (1936) performs the reaction in a slightly different way and

claims that it demonstrates the presence of phenolic compounds, which would give an orange, red or blue coloration, a yellow color being only due to azoproteides without special significance.

### THE CHARACTERIZATION OF BASIC AND NON-BASIC PROTEINS

The arginine reaction is suitable not only for the characterization of proteins in general, but also for giving an idea of the relative richness of the proteins in this amino acid. As the coloration is more or less proportional to the arginine present (Serra, 1944b) a very intense reaction demonstrates the existence of proteins, such as the histones, with a basic character due to the excess of basic amino acids and especially of arginine. Besides the histones, only the protamines and the edestins possess a great amount of this amino acid; but as the edestins are plant globulins found only in seeds and the protamines have been encountered chiefly in fish sperms, in general the presence of a high concentration of arginine can be interpreted as indicating that the proteins are of a basic type resembling the histones.

On the other hand, a weak arginine reaction may be taken as indicating the absence of basic proteins, or at least that these can be present only in small amounts. In this case, the proteins are of the non-basic type.

Within the non-basic proteins, it is yet possible to distinguish several protein classes by their solubility. Thus, the albumins are soluble in water, while the globulins dissolve only in diluted salt, acid and alkaline solutions, and the scleroproteins, like the keratins and collagen, require strong alkali to go into solution. Though these distinctions can be clearly seen in test tube assays, the conditions in the cell do not in general allow conclusions to be reached by this method; nevertheless, the solubility has been used in cytophysiological work (for instance by Gersch, 1940).

When interpreting histochemical reactions quantitatively it is always necessary to bear in mind that certain assumptions are implicitly made, namely (1) that the color intensity is proportional, even only roughly, to the quantity of the compound which is present in the cell formation; (2) that the density of structure, or relative concentration of substance, is not very different in the parts which are being compared, for instance in the ground cytoplasm and the nucleus; and (3) the method must not give rise to secondary color impregnations or adsorptions. Condition 3 is experimentally controllable, while questions 1 and 2 are in general more or less a matter of critical judgment. But if color intensities, which in test tube assays are known to be more or less proportional to the concentration,

are very intense in a certain cell structure, say four or five times more intense in the chromosomes than in the ground cytoplasm, we may safely conclude that the relative concentration is greater in the chromosomes, because the difference in density of structure must not be so great. It is, however, always necessary to be critical about these results. The ultraviolet microspectroscopy can also be affected by condition 2.

The color intensity may be roughly measured by comparison of the apparent coloration seen at a given microscope magnification, with color charts like those used by Levine (1940). Successive dilutions of a certain dye, whose color matches well the color seen through the microscope, are prepared and painted on small squares on white drawing paper. Alternatively, the comparison can be made by means of a visual colorimeter like the Tintometer (Salisbury, England), with logarithmically graduated glasses, the tube of the colorimeter being placed close to the eyepiece of the microscope (Serra and Queiroz Lopes, 1944a).

#### THE CONCENTRATION OF PROTEINS IN GENERAL AND THE ZONES OF INTENSE METABOLISM

While the arginine reaction may be used quantitatively to characterize the basic proteins, the reactions for tyrosine, for tryptophane and for the protein SH groups can serve as indications respectively of the relative richness of the cell structures in proteins in general, or of the metabolic activity of a given formation.

Tyrosine seems to be present in almost all natural proteins in amounts which are not very different for the various classes, excepting principally silk fibroin, pepsin and insulin (see the tables of Calvery, 1938; and Lloyd and Shore, 1938). We can say that in the great majority of cases a strong tyrosine reaction indicates a great richness of proteins in general, and if the density of structure is not very different, the intensity of the reaction can serve as a rough quantitative estimation of the proteins present in several structures.

Tryptophane is also a constituent of the great majority of the proteins, generally in small amounts between 1 and 3%. The histones cannot be distinguished by means of their content in this amino acid. A more intense reaction can have the same significance as the tyrosine reaction, or it can be taken as suggesting the existence of a zone of intense metabolism in which peptides are being synthesized or split (Serra and Queiroz Lopes, 1945).

An intense reaction for protein SH groups (that is, for sulfhydryl groups which remain after extraction of the glutathione) has also been

supposed to demonstrate the existence of active metabolic and formative changes in the proteins (Brachet, 1940). In fact, it is known that denaturation of proteins is accompanied by an increase in reactive SH groups, and this is supposed to be due to an unfolding of polypeptide chains. It is, therefore, probable that an intense reaction for protein sulfhydryl groups might be taken as indicating an active protein synthesis or breakdown. As we have emphasized above, it is also necessary to be critical in this case, when using these reactions for quantitative purposes.

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# A RAPID HISTOLOGICAL TECHNIC FOR STAINING LATEX IN ROOTS OF TARAXACUM KOK-SAGHYZ<sup>1</sup>

LUCY B. ABBE<sup>2</sup>

**ABSTRACT.**—The freezing technic described in this paper provides permanent slides of sections of the root of *Taraxacum kok-saghyz* with latex preserved in place. The following schedule is used: (1) Prefreeze the piece to be sectioned before removal from the root. (2) Mount in ice and section with chilled microtome knife. (3) Plunge frozen sections into the combination coagulant and stain prepared from Calco oil blue N. A., acetic acid and ethyl alcohol. (4) Wash in water. Aspirate if necessary. (5) Mount on a slide using Karo.

This technic is rapid and simple. The sections are well adapted to making counts and measurements of latex tubes since there has been a minimum of latex loss. Latex is retained in place by keeping tissues frozen until introduced into the coagulant.

## INTRODUCTION

Frozen sections have been useful for a study of the latex system in roots of *Taraxacum kok-saghyz*. In this study it was desirable to obtain permanent mounts of uniformly thick sections with the latex preserved and stained in place. It was also important to find a rapid technic which could be learned quickly by unskilled workers. For this reason all unnecessary steps were avoided in favor of speed and ease of handling.

## PROCEDURE

1. *Freezing* — To prevent loss of latex the part of the root to be sectioned is frozen quickly and is kept frozen until the sections are allowed to thaw in the combination stain and coagulant. From the

<sup>1</sup>Cooperative investigations by the Rubber Investigations, Bureau of Plant Industry, United States Department of Agriculture, the Botany Department and the Division of Agronomy and Plant Genetics of the University of Minnesota.

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The writer is grateful to Miss Olga Hansen of the Botany Department for valuable suggestions concerning the technic; to Dr. E. C. Abbe of the Botany Department, Dr. Hayes of the Division of Agronomy and Plant Genetics and Dr. W. G. Whaley of the Division of Rubber Plant Investigations, for critical reading of the manuscript; and to R. W. Henderson of the Division of Rubber Investigations, under whose direction the work was done, for making the rubber determinations and calculating the correlation co-efficient.

whole root or a piece of root at least 4 or 5 cm. long, a segment of root about 1 cm. in length is frozen quickly with dry ice (solid  $\text{CO}_2$ ) or liquid  $\text{CO}_2$ . The time required for freezing depends mainly on the diameter of the root. Using solid  $\text{CO}_2$ , 5–10 seconds was required to freeze properly a root 3 mm. in diameter.

2. *Mounting for Sectioning* — A segment of well-frozen root 5 mm. in length is then mounted on a dry ice or liquid  $\text{CO}_2$  freezing attachment. Care should be taken not to let the root thaw during the above process, in which case it will turn brown and the latex will form adhesive strands which stick to the knife and pull out during sectioning.

3. *Sectioning* — A sliding microtome is used for sectioning. The microtome knife may be kept at freezing temperature by fastening a piece of solid  $\text{CO}_2$  above the cutting edge. The root segment should be frozen to the point where it is firm but not brittle. If the root is frozen too hard or if the knife is too cold the sections will crack. Sections may be cut 20–100  $\mu$  thick but should be of uniform thickness for comparative studies of the latex tubes. Sections are removed from the knife with chilled forceps and plunged into the combination coagulant and stain where they are allowed to thaw. Preliminary extraction of lipids, resins and similar compounds did not seem essential.

4. *Staining* — The stain is made as follows: One-half gram of Calco oil blue N. A. is added to 100 ml. of 50% ethyl alcohol. Shake well and allow to stand 48 hours. Part of the dye will dissolve. Of the resulting clear blue solution of Calco oil blue 95 ml. are then decanted, filtered, and mixed with 5 ml. of glacial acetic acid in stock bottle 2. The primary function of the acetic acid is to help coagulate latex.

The staining solution is used only while it remains clear. If it becomes slightly cloudy, staining will be poor and the sections will be covered with dye crystals which are difficult to remove.

Staining for 45 minutes is usually sufficient to give a bright blue stain to the coagulated latex (Fig. 1). The stained sections should be rinsed quickly in distilled water before mounting.

Whittenburger (1944), Artschwager (1943) and Addicott (1944) have previously discussed the use of Calco oil blue as a latex stain.

5. *Mounting* — Sections are mounted in a drop of a solution of 0.1% carbolic acid, 66 $\frac{2}{3}$ % high grade white Karo, and 33 $\frac{1}{3}$ % water. The Karo solution dries to a fine hard mount in a reasonably dry atmosphere, and has been useful when alcohols and xylenes are to be avoided during mounting. The use of Karo is previously mentioned by Johansen (1940, see p. 24), Patrick (1936), and Addicott (1944).

If trapped air bubbles are objectionable, they may be removed by aspiration while the sections are being washed.

Slides made by the above method have remained in excellent condition for almost two years.

*Practical Application of the Method* — Sections made by the above method have been used to determine the degree of correlation be-

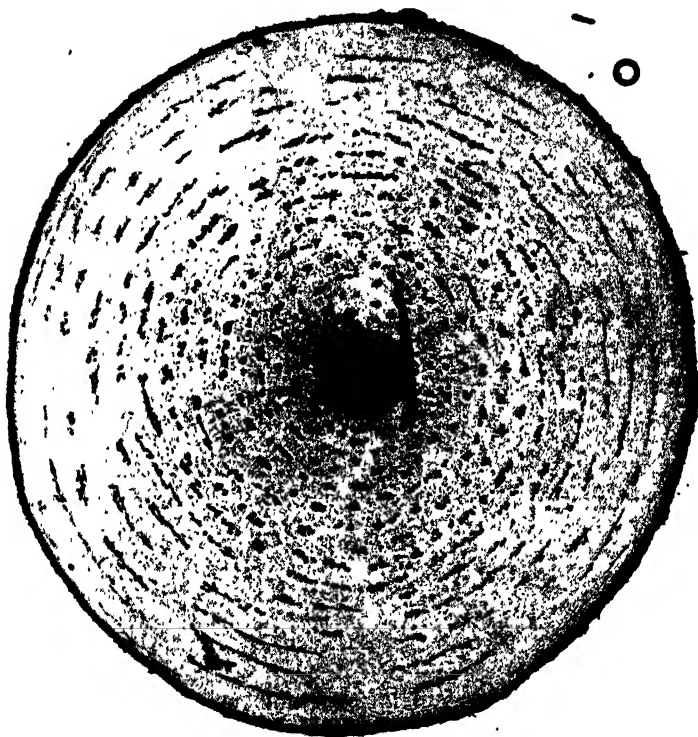


Fig. 1. Cross-section of *Taraxacum kok-saghyz* root showing darkly stained latex strands in the concentric rings of latex tubes.  $\times 22$ .

tween rubber percentage and certain characteristics of the laticiferous system in the root of *Taraxacum kok-saghyz*.

That latex and latex tubes are well preserved by this method is indicated by the high degree of correlation between the per cent of rubber in the roots and visual ratings for latex density of sections taken from the same roots. Using 90 roots from as many different plants, the correlation between the latex density ratings and the

per cent of rubber in the same roots was .753. This greatly exceeds the correlation coefficient of .267 required for significance at the 1% level.

The chief advantages of the method described in this paper are (1) retention of relatively normal cell size and (2) ease and speed with which sections may be prepared.

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## A GOLD CHLORIDE METHOD FOR MOTOR END PLATES

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Since Ranvier<sup>1</sup> established the method of precipitating a metallic salt in nervous tissue by means of an acid, this technic and modifications of it have been used extensively. However, in the staining of terminal axons and the motor end plates of striated muscle, the results have too often been erratic. The method herein described eliminates much of the variation due to the solutions used; it has been used with uniform results in the preparation of several thousand teased specimens.

Three solutions are used in this staining method:

- (1) Citric acid in solution—10% citric acid in normal saline solution.
- (2) Gold chloride,<sup>2</sup> 1% (1 gram gold chloride in 100 ml. distilled water). This solution should be at least 24 hours old for good results. It does not deteriorate with age. The gold chloride solution can only be used once.
- (3) Aqueous formic acid 20%.

### METHOD

1. Remove striated muscle and immerse in 10% citric acid. Let the tissue remain in this solution for a minimum of 10 minutes and a maximum of 30 minutes.

2. Place directly (without washing) in the 1% solution of gold chloride. Let the tissue remain in this solution for about 60 minutes, or until the color of the tissue is a dark yellow. It is not necessary to keep the solution in the dark. However, direct sunlight is detrimental to the staining process. For best results, tissue should not be greater than 4 mm. along two edges when placed in the gold chloride. If it is necessary to use larger pieces of tissue, the time in solution may be proportionally increased.

3. Without washing, place tissues in a 20% solution of formic acid. Allow the tissue to remain in this solution a minimum of 10 hours and maximum of 20 hours.

4. Place tissue without washing in a 50% mixture of 95% methyl alcohol and pure glycerin for several hours. Remove the top of the

<sup>1</sup>Ranvier, R. 1880. On the terminations of nerves in the epidermis. *Quart. J. Micr. Sci.*, 80, 456.

<sup>2</sup>Gold Chloride, auric, Mallinckrodt Chemical Works, St. Louis, Mo.

container and when the alcohol has evaporated transfer the tissue to pure glycerin. The tissue will keep in glycerin for months without deterioration.

To mount, tease muscle fibers and place on slide in a drop of pure glycerin. Place cover glass over the teased muscle and seal with 60% Clarite solution, a synthetic resin,<sup>3</sup> or Harkite, a synthetic resin in toluene.<sup>4</sup> The evaporation of the alcohol in step 4 helps to prevent the formation of air bubbles in the finished slide. By using small pieces of tissue, artifacts, such as air bubbles and distorted muscle fibers, are prevented.

This method results in permanent sections. The muscle fibers stain red-blue while the motor end plate and medullated axon stain black (Fig. 1). The nuclei of the muscle fibers do not stain with this technic, but the striations of the muscle fibers are very well demonstrated. Under favorable circumstances this method also demonstrates myoneural bundles and neurotendinous endings.

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<sup>3</sup>Central Scientific Co., New York, N. Y.

<sup>4</sup>Hartman-Leddon Co., Philadelphia, Pa.

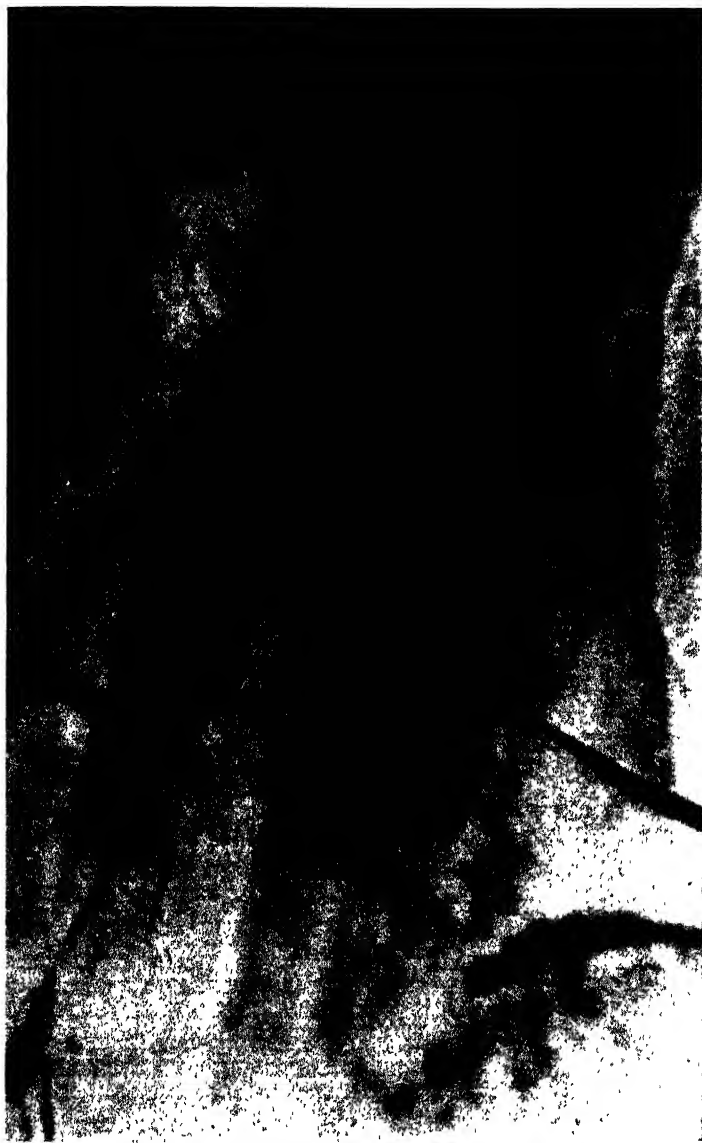


Fig. 1. Motor end plate (rat). Modified gold chloride.  $\times 940$  and enlarged  $\times 3$



## DEMONSTRATION OF THE GROUND SUBSTANCE OF CARTILAGE, BONE, AND TEETH

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**ABSTRACT.**—A slab of bone about 5 mm. thick is decalcified in 5% HCl, washed, and placed for several days to 2–3 weeks in 3% KOH in 20% glycerin (if the bone is medium sized); for small bones the KOH should be decreased to 1%, and for large bones it may be increased to 5%. The solution is changed frequently. When the bone begins to dissociate, it should be removed and washed in water till all traces of alkali are removed. The specimen is passed through 3 changes of dioxane into paraffin, and then through a second paraffin bath into the final paraffin. Sections are cut at 10–12  $\mu$  and stained with VanGieson's picro-fuchsin or with orcein.

Several techniques for unmasking the fibrillar ground structure of the supporting tissues have been described in the literature, but the results obtained by these methods have proved disappointing. In attempting to display the fibrillar structure of bone, the method herein described has proved satisfactory in this laboratory, and has been applied to the study of the structure of teeth and cartilage also, with instructive effects.

Digestion of tissues by ferments and alkalis for the study of structural details that otherwise remain masked is a recognized technic. It has been applied in one form or another to the study of practically every tissue in the body. The following procedure depends upon KOH in aqueous solutions of different strengths to wash out the amorphous interstitial substance and unmask the fibrillar structure of the supporting tissue in question. Bone is used here, but teeth and cartilage are treated in the same manner.

A slab of bone about 5 mm. thick is decalcified in 5% HCl. Bone in any condition may be used—fresh, desiccated, fixed, or unfixed. Fresh, unfixed bone seems to respond best to this treatment. If the specimen is greasy, it may be degreased with a suitable fat solvent (acetone, ether, benzene, or gasoline are all satisfactory) before decalcification.

Decalcification is followed by washing to remove the acid, and the slab then is treated with a KOH solution made as follows (Mall, 1906):

Water	77 ml.
Glycerin	20 ml.
KOH	3 g.

This treatment is continued until signs of dissociation appear, and may require from several days to two or three weeks, according to the structural nature of the ossein in a given species. Experience will show that this varies in different animals, and a single rule cannot be formulated to cover all cases. The solution should be changed as often as it begins to appear cloudy. Signs of dissociation are recognized by a fuzzy appearance of the edges of the tissue, or by the inception of actual fragmentation. When this begins, the specimen should be removed immediately to water, and should be washed until all traces of the alkali are removed—preferably in running water overnight. In some cases a certain amount of disintegration of the tissue is necessary before the proper amount of the amorphous interstitial substance has been removed and the fibrillar structure becomes clearly apparent. Again experience must dictate the extent to which this should be carried.

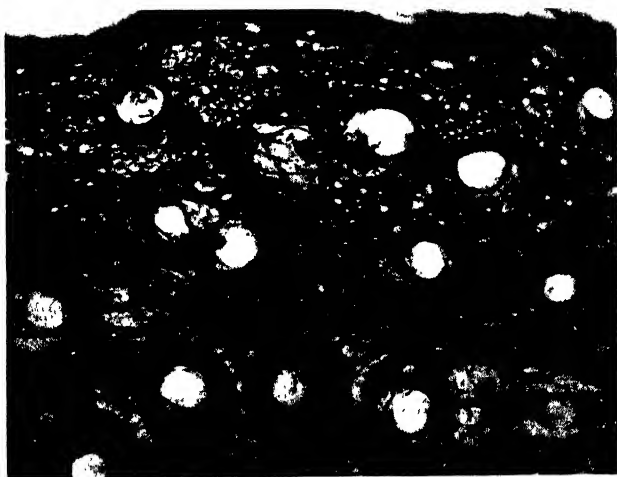
The discussion thus far applies to slabs of medium-sized bones; i. e., bones of the size of the femur of the rat to those of the size of a dog femur. Smaller bones, and delicate bones with a very thin cortex are treated with the same reagent, but with the KOH reduced to make a 1% solution. If such bone shows no change after a week of this treatment, it may then be transferred to the more concentrated potash solution. Large bones (i. e., femora of human adults and larger) may need more vigorous treatment. After an initial period in the 3% potash solution these specimens may be transferred to a 5% solution, if they have not shown indications of dissociation. In the stronger concentration the tissue must be watched carefully, as changes may occur rapidly and destroy the ossein completely.

Thorough washing of the macerated slab is followed by embedding in paraffin for sectioning. The specimen is passed from water into pure dioxane, using three changes, with one hour in each, for medium-sized and small bones. Slabs from large bones may be left in dioxane for a longer time for complete dehydration, without danger of too much hardening. From dioxane the bone is passed into melted paraffin—one hour in each of three paraffin baths, or until all dioxane has been removed. It is well to stir the paraffin occasionally, as the dioxane does not diffuse readily.

Sections cut at  $10\mu$  to  $12\mu$  are satisfactory. They may be stained with Van Gieson's picro-fuchsin, or with orcein. The synthetic orcein produced by MacAndrews and Forbes Company, Camden, N. J.<sup>1</sup>, gives the best results. The bones of animals having Haversian systems show an unusual effect in that the fibrillae of the alter-

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<sup>1</sup>Now sold by Eastman Kodak Co., Rochester, N. Y.

**Fig. 1****Fig. 2**

**Fig. 1.** Section of a Haversian system of a human femur, stained with orcein. This type of bone shows a regularity in orientation of fibrillae, and the diffuse lamellae are stained selectively with orcein. With picro-fuchsin the compact lamellae are stained with fuchsin. Mag. 90 $\times$ .

**Fig. 2.** Section through the cortex of the femur of a pigeon. Fibrillar structure is less regularly organized, and does not stain differentially. Dissociation has been carried considerably farther than in Fig. 1. Mag. 200 $\times$ .

nating compact and diffuse lamellae stain differentially with these stains (Fig. 1), as described elsewhere (Ruth, 1945), when the proper degree of dissociation has been effected. Bones of animals in which the fibrillar structure is organized less regularly (Fig. 2) do not exhibit this differential staining reaction. The gradual fading of the Van Gieson stain is annoying, and may be compensated for by the use of the Ponceau S mixture of Curtis (Carleton, 1938, p. 109). It is said to have the disadvantage of not being as specific for newly formed connective tissue, but it is a permanent stain. Ponceau S is not listed by Conn (1940), and Ponceau B (syn: Biebrich scarlet, water sol.; croceine scarlet; scarlet B, or EC; and double scarlet BSF) has been used in its place.

#### SUMMARY

A method is described for demonstrating the fibrillar structure of decalcified bone by washing out the amorphous collagen with a 3% KOH solution to which some glycerin is added. When dissociation of the ossein has progressed far enough the specimen is embedded in paraffin; and sections are cut at  $10\mu$  to  $12\mu$ . The fibrillae are stained with orcein or picro-fuchsin. These stains show a selective specificity that permits a differential staining of the diffuse and compact lamellae in bones having Haversian systems. Bones that do not show such a well-oriented fibrillar structure do not have this differential staining reaction. The use of Ponceau B instead of fuchsin in Van Gieson's stain is suggested to circumvent the tendency of the stain to fade.

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## CARBOL CRYSTAL VIOLET<sup>1</sup>

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There is some call among bacteriologists for a "carbol gentian violet". It is used sometimes as a general bacterial stain and sometimes in the Gram technic, to be followed by treatment in iodine and decolorization. The use of the formula is generally ascribed to Nicolle (1895), although it is far from certain that he originated the idea. Inasmuch as carbol fuchsin had then been in use for at least a decade, it would be surprising if no one had prepared a similar "gentian violet" formula before 1895. Although a search of the literature would probably bring out some such formula earlier than the paper of Nicolle, none has yet been found, and it has hardly seemed an important enough point to make the necessary search.

The formula first came into use as improvement on Ehrlich's "anilin gentian violet"; and there can be no question but that it is an improvement. It seems to be an equally efficient stain, to work practically the same in the Gram method, and to have the advantage of greater permanency of the solutions. In fact, a properly prepared carbolic crystal violet seems to be about as permanent as a staining fluid can be made; while the anilin-water formula is notoriously unstable. Nevertheless, the writer has never recommended (either personally or on behalf of the Committee on Bacteriological Technic) the use of carbol crystal violet in the Gram stain; the ammonium oxalate formula (Hucker, 1922) has always seemed, and still seems, to be a distinctly more satisfactory staining fluid for the purpose.

The only reason for the present paper on carbol crystal violet is that recent correspondence brings out two facts: first, that in some quarters the formula is in use; second, that difficulties are experienced when making it up either by Nicolle's original formula or by the emended formula recommended by the Committee on Bacteriological Technic. The difficulty frequently observed is that the solutions sometimes tend to gelatinize, often becoming semisolid before they have stood 24 hours.

The original formula of Nicolle called for one volume of saturated alcoholic "gentian violet" to 10 volumes of 1% aqueous phenol. It was found some 20 years ago that satisfactory results were obtained with this formula when approved samples of crystal violet, such as

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<sup>1</sup>Journal Paper No. 662, New York State Agricultural Experiment Station, Geneva, N. Y., January —, 1946.

then were on the market, were used for the dye. These samples had a dye content of about 85%. The objection to the formula was that it was indefinite, even when crystal violet was substituted for the indeterminate "gentian violet" originally called for, because a saturated alcoholic solution of crystal violet cannot be regarded as containing a fixed quantity of the dye. Hence an effort was made to state it more definitely by emending the formula to call for a 1% solution in ethyl alcohol of a sample having 85% dye content. Such solutions made up at that time were stable; this proves now to be the case with at least one recently manufactured lot of crystal violet having a dye content of only 88%.

The majority of present-day crystal violets, however, have a dye content of 90 to 95%, and with them the formula in question is less satisfactory. Just why the objectionable gel should form with a 1% solution of one of the present-day more concentrated batches of the dye but not with a saturated solution of the weaker dyes of twenty-odd years ago, is difficult to explain; but it is certainly true that when a 1% solution of one of the batches now on the market, having a dye content of over 90%, is mixed with a 1% aqueous solution of phenol, the fluid gelatinizes when allowed to stand overnight.

The difficulty can simply be avoided by using a crystal violet solution something less than half as strong as that originally recommended. Accordingly the formula now recommended is:

Solution A		Solution B	
Crystal violet CC <sup>2</sup>	0.4g.	Phenol	1g.
Ethyl alcohol (95%)	10. ml.	Distilled water	100ml.

#### Mix solutions A and B

This formula is recommended merely as an improvement on the Nicolle formula, when carbol crystal violet is desired. It must be distinctly understood, however, that the writer still regards the ammonium oxalate formula as preferable, both in the Gram technic and for ordinary bacterial staining.

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<sup>2</sup>i. e. Commission Certified.

## LABORATORY HINTS FROM THE LITERATURE

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A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS  
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

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### MICROSCOPE AND OTHER APPARATUS

#### A. B. The Maltwood finder. *Microscope*, 5, 268. 1945.

The usual type of this finder takes the form of a ruled mesh 50 units square (2500 units). Each unit bears 2 numbers indicating its position relative to the left-hand and upper margins respectively. Thus the third square in the second row is marked 2<sub>3</sub>. The slide (preparation) to be searched is examined with a suitable combination of objective and ocular and moved by means of a mechanical stage or other device, the slide being kept firmly against the stop. When the object whose position is to be recorded is located, the slide is carefully removed and the finder is inserted in its place. The numbered square visible in the field is noted.—C. E. Allen.

#### BROWNELL, CHARLES G. Simplified micro-projection for small groups. *J. Techn. Methods*, 24, 51-4. 1944.

A monocular microscope and a standard microscope lamp are employed for micro-projection. The microscope is placed in a horizontal position on a table with a lamp behind it, arranged so that the distance from the small hole of the closed lamp diaphragm to the table is equal to that of the closed substage diaphragm to the table. The Kohler method of illumination is recommended. By this method an image of about 24 inches in diameter can be focused on the screen.—Challiss Randall.

#### BURKE, FREDERICK. New form pipette. *Microscope*, 5, 252. 1945.

One end of a capillary tube (1 mm. bore) is closed and blown out to form a short bulb. The outer half of the bulb is strongly heated and blown off, leaving a short conical end. To this end is sealed a larger bore tube fitting over and extending beyond the opposite end of the smaller tube. A rubber nipple is fitted over the end at which the two tubes are sealed. The device is recommended for handling mosquito and other aquatic larvae.—C. E. Allen.

#### KELEMEN, GEORGE. Microscopy and photomicrography of the temporal bone and polarizing films. *J. Biol. Photog. Assoc.*, 13, 171-6. 1945.

Polarized light is a great aid in analyzing structural details in general bone histology under normal and pathological conditions. Unfortunately, however, polarizing apparatus is expensive and not always available, but in the greater part of the examinations, it can be replaced by "Polaroid" polarizing films which offer a method of extreme simplicity at negligible expense. A piece of polarized film of the same size as the slide is clamped to the stage of the microscope under the slide carrying the unstained section. This piece serves as a polarizer. A second small round piece is dropped onto the inner diaphragm of the ocular. This serves as an analyzer. By rotating the ocular, opaque and bright positions are produced.

For photomicrography a round piece of the film is fastened in front of the objective and a second piece is slipped into a filter carrier frame between the microscope and the source of light. The second piece is easily rotated. This method is especially desirable because the greatly restricted visual field resulting from the small size of the Nicol prisms is avoided. The author includes photomicrographs to show the use of polarizing film in routine histological analysis of temporal bone.—Jean E. Conn.

## MICROTECHNIC IN GENERAL

**DE ANGELIS, EUGENE.** The use of plastics to replace cover glasses in microscope slide preparations. *J. Lab. & Clin. Med.*, 30, 469-71. 1945.

Tissues or other slides ready for mounting are dipped in the plastic for 90 sec., removed, allowed to drain, and dried at room temperature. If the slides are to be filed, they are dried in an oven at 140° C. for one hour. The most satisfactory plastics tried were Acryloid B-72 (Resinous Products and Chemical Co., Philadelphia, Pa.) and Vinylseal (Bakelite Corporation, New York, N. Y.) These adhered well to glass and tissue, had the maximal optical transparency, were resistant to abrasion and common solvents, showed negligible deterioration by light and chemicals, and were easy to apply.—*John T. Myers.*

**HALL, WILLIAM E. B.** A method for the preparation of gross demonstration and museum specimens using cross-sectioned tissues. *J. Techn. Methods*, 24, 12-20. 1944.

The author describes a method of preparing gross demonstration specimens which consists of placing properly fixed slices of tissue between two sheets of glass, immersing in molten paraffin or paraffin-tissuemat mixture, cooling, and appropriately sealing. Both Nile blue sulphate and scarlet red (Sudan IV) are recommended as fat stains. The technic for Nile blue sulphate is as follows: stain section tissue slices in saturated (less than 2%) aqueous Nile blue sulphate, 15-20 min.; rinse in water; differentiate in 1% aqueous acetic acid, 10-20 min.; wash thoroughly through several changes of distilled water, 1-2 hr.; moisten surface with 4% aqueous formaldehyde. The following technic is advised if Sudan IV is used: moisten briefly in 70% alcohol; immerse in Herxheimer's scarlet red solution, 1-2 min., keeping sections in slight motion; wash briefly in 70% alcohol; wash well in mixture of 1 part 95% alcohol and 1 part 4% formaldehyde until non-fatty tissue becomes white; drain; wash briefly in 4% aqueous formaldehyde. Because of the affinity of this stain for paraffin as well as for fat, speed in the embedding and subsequent hardening is essential and every effort possible should be made to keep the border of the specimen moistened.

Marchi's method, Weigert's method, and LeMasurier's method are all recommended as stains for cerebral and nervous tissue, hematoxylin and Nile blue sulphate for tissues which consist of or include bone.—*Challiss Randall.*

**LILLIE, R. D.** Picroformaldehyde formic acid for fixation. *J. Techn. Methods.*, 24, 35-6. 1944.

Picro-formalin-formic acid is a modification of Bouin's fluid which contains 5 parts formic acid, 10 parts strong formaldehyde, and 85 parts saturated aqueous picric acid solution. One change of 70% alcohol is used, then 80%, and the usual acetone gasoline sequence with vacuum infiltration in paraffin. For Romanovsky staining, sections are washed 5-10 min. to remove picric acid. This solution is a good decalcifying agent and avoids over-fixation of the surface layer of solid organs. Otherwise the general effect is similar to that of Bouin's fluid.—*C. Randall.*

## DYES AND THEIR BIOLOGICAL USES

**ALBERT, A., and GLEDHILL, W.** Improved syntheses of aminoacridines. Part IV. Substituted 5-amino-acridines. *J. Soc. Chem. Ind.*, 64, 169-72. 1945.

The reaction whereby 5-chloroacridine is converted into 5-aminoacridine was applied to 24 variously substituted 5-chloroacridines with generally good results, giving the correspondingly substituted 5-aminoacridines, many of which have proved to be powerful antibacterials.—*R. T. Whittenberger.*

**MILLIGAN, R. F., and HOPE, F. J.** The structure of dichlorofluorescein. *J. Amer. Chem. Soc.*, 67, 1507-8. 1945.

D and C orange No. 8, a new dye permitted by the U. S. Government for use in the manufacture of drugs and cosmetics, was shown to be dichlorofluorescein with the chlorine atoms occupying the 4 and 5 positions in the molecule.—*R. T. Whittenberger.*

WOOD, HARLAND G., and RUSOFF, IRVING I. The protective action of trypan red against infection by a neurotropic virus. *J. Exp. Med.*, 82, 297-309. 1945.

Trypan red, when injected intraperitoneally into mice, was found to lower greatly the incidence of infection of mice inoculated intraperitoneally with a virus similar to that of poliomyelitis. The protective action of the dye is overcome if the virus is inoculated in too high concentration. The lowered incidence of infection was observed in mice inoculated with virus as long as 29 days after the last dye injection. Brilliant vital red and Congo red were also found effective. In cotton rats similarly inoculated, trypan red had a similar effect. With monkeys and a typical poliomyelitis virus, no protection was observed, but the experiment was considered inadequate.—H. J. Conn.

### ANIMAL MICROTECHNIC

HINER, R. L., MADSEN, L. L., and HANKINS, O. G. Histological characteristics, tenderness, and drip losses of beef in relation to temperature of freezing. *Food Research*, 10, 312-24. 1945.

Histological sections of meats frozen and examined within 24 to 36 hr. were studied by the following technic:

Frozen blocks of beef were sectioned by a sliding microtome, precooled in a room maintained at approximately  $-7.8^{\circ}\text{C}$ ., unrolled carefully on a cold slide, thawed by warming the slide from beneath with the operator's fingers, and then placed directly in 70% alcohol, or passed through graded alcohols of 30 and 50% concentration, for about two hours in each, before placing in 70% alcohol. The sections remained in 70% alcohol overnight, then were stained with hematoxylin and eosin, and mounted in the usual manner. Alcohol fixation caused some shrinkage which could be reduced by dipping the sections in 80% alcohol for 3 or 4 sec. to fix the tissue to the slide and then withdrawing and placing in 50% dioxane for 24 hours, after which they were placed in absolute dioxane for another 24 hour period. This latter procedure was followed by the usual staining and mounting technic and was believed to preserve more nearly the original structure of the fiber.

Macroscopic observations of tissues confirmed changes noted in the study of the frozen sections. As freezing temperatures were lowered, precipitated proteins and nuclear fragments outside the fibers became more extensive. Intrafibrillar ice crystals and areas, with fiber-splitting caused by ice formation, were clearly shown.

Frozen meat, thawed, fixed in formalin, dehydrated in dioxane, embedded in paraffin, sectioned and stained, did not preserve the detail observed by the methods described above.—William G. Walter.

HOLCOMB, R. C., and APTEKMAN, PAUL M. A method of preparing fetal bone for exhibition and ready examination by methacrylate. *J. Techn. Methods*, 24, 21-4. 1944.

The authors describe a technic by which frail fetal bones may be embedded in plastic without distortion. The bone specimens may be stained with alizarin red S and the stain will remain unaltered by the method described. The monomeric ethyl methacrylate used is a clear plastic which transmits every color of the spectrum and which has a light transmission value of 91%. The resin is partially polymerized before the specimen is embedded in it. Polymerization is completed by oven treatment at  $55-65^{\circ}\text{C}$ . for 48 hr. Though some shrinkage occurs, there is no distortion in the specimens.—Challies Randall.

LENDRUM, A. C. On the "pink" epithelium of the cystic breast and the staining of its granules. *J. Path. & Bact.*, 57, 267-70. 1945.

Granules in the epithelium of the cystic breast may be demonstrated by the following method: Fix tissue in formol-sublimate; cut paraffin sections as usual. Stain with Mayer's hemalum. Then stain 1-2 min. in 1% aqueous fast green containing 0.5% acetic acid. Rinse in water and immerse in Lugol's iodine 2 min. Rinse in 95% ethanol. Immerse in 2% phosphotungstic acid in 95% ethanol. Rinse briefly in water. Stain in "carbacid fuchsin" 2-6 min., con-

trolling under the microscope. To prepare "carbacid fuchsin", melt 0.4 g. phenol and mix with 1.0 g. acid fuchsin. When cool dissolve in 10 ml. 95% ethanol and make to 100 ml. with starch-dextrin (grind 0.5 g. starch to fine powder, then grind in 0.5 g. dextrin, add gradually with grinding 100 ml. water and heat to 80° C.). When cool filter into the phenol sludge solution. The granules stain blackish-red as a result of taking up some of the fast green together with the subsequent fuchsin.

As the nuclear stain is removed in part by the subsequent treatment with iodine, greater nuclear detail is obtained by staining, prior to the hemalum, with celestin blue (dissolve 2.5 g. iron alum overnight at room temperature in 50 ml. distilled water, add 0.25 g. celestin blue R, C.I. 900, and boil 3 min. Filter, add 7 ml. glycerol).

The green stain is partly removed by iodine. Stronger final green staining of cytoplasm is obtained by increasing the original time of staining. The time in iodine should not be less than 2 min.—S. H. Hutner.

**LENDNUM, A. C., CARSON, W., and PENNY, W.** On the staining of basement membranes. *J. Path. & Bact.*, 57, 270-1. 1945.

The method devised for staining of granules (see previous abstract) was found to give excellent staining of the basal membrane of the renal glomerulus provided the following additional step is performed: Subject paraffin sections before de-waxing to the vapor of hot formalin (heat formalin in a closed dish at 55-65° C. for about 2 hr.). Then stain as already described.—S. H. Hutner.

**LILLIE, R. D.** Study of certain oil soluble dyes for use as fat stains. *J. Techn. Methods*, 24, 37-42. 1944.

Satisfactory fat staining in which lipids are preserved may be obtained in 2 to 5 min. by using a 30:30:40 or 25:25:50 solvent of methyl or ethyl alcohol, acetone and water saturated with Sudan brown (C. I. No. 81), Sudan R (C. I. No. 119), Sudan brown 5B, oil red 4B, oil red EGN, Sudan II (oil red O), (C. I. No. 73) or Sudan black B. Precipitation may be prevented by dipping the sections in the same solvent mixture as used for the dye for 2-3 sec. just before staining. The Sudan browns give a deep brownish red, the oil reds and Sudan R a deep orange red, the Sudan II a light yellowish orange, and the Sudan black B a greenish black. Other good dyes are Sudan III (C. I. No. 248), oil brown M, Sudan Corinth B, and Sudan red 4BA.—C. Randall.

**LILLIE, R. D.** Various thiazin and fluorane dyes in paired combinations as neutral stains for tissue. *J. Techn. Methods*, 24, 43-5. 1944.

A study was made of various commercially available thiazins and eosins as components of the neutral stain in the buffered Romanovsky technic. Both Nocht and Giemsa type technics were used. Sections were brought from paraffin to water in the usual way, stained 1 hr. in the appropriate mixture, rinsed in water, dehydrated with acetone, passed through acetone and xylene into xylene and mounted in clarite. Eosinates were made and 0.2 g. portions dissolved in 100 ml. of a mixture of equal parts of C.P. glycerol and methanol which had been redistilled from silver oxide. Sections were stained at pH 4.6 for 1 hr.

The author recommends the B and Y eosinates of azure A, azure C and toluidine blue.—C. Randall.

**NEUDA, PAUL M., and ROSEN, MAURICE S.** A preliminary report on a rapid method for diagnosing sickle-cell disease. *J. Lab. & Clin. Med.*, 30, 456-8. 1945.

Sickle cell forms appear promptly when a drop of sickle-positive blood is in contact with a broth culture of normal human feces. The procedure used is as follows: Mix a cherry-size piece of feces with 5 ml. of isotonic NaCl solution, stir and filter. Add 0.1 ml. of the filtrate to a tube of nutrient broth and incubate 24 hr. at 37° C. Place a drop of this culture on a slide, add a drop of the suspected blood, apply a cover slip and examine microscopically. There is an agent in the broth which causes susceptible cells to rapidly assume the sickle form.—John T. Myers.

**ZIEGLER, J. A.** Use of the benzidine staining method for the study of capillaries in the cornea. *Canad. J. Res., Sec. E*, 23, 115-17. 1945.

In a study of the metabolism of riboflavin, it was necessary to study the capillaries of rat corneas. As other methods failed to differentiate the vessels from the remainder of the corneal tissue, the following technic was devised: remove the whole eye and fix in 10% formalin-saline fixative, 2 hr.; fix in fresh fixative, not less than 1 week; wash eye; excise cornea; cut cornea in four equally spaced centripetal incisions; flatten out cornea; wash in frequently changed distilled water, 30 min.; stain in freshly prepared Solution A, 20 min.; wash in distilled water, 10 to 15 sec.; place in Solution B, 15 min. at room temp. and then 30 to 45 min. at 37° C.; wash in distilled water, 15 min.; dehydrate in 70 and 95% alcohol, each acidified with 2% acetic acid; dehydrate in neutral 100% alcohol; clear and mount in sandrac, camsal, dioxane mixture. Stained corneas must not be unduly exposed to light. Blood vessels filled with blood are jet black, those not filled are faintly brown, and the background is pale yellow. Solution A is a mixture of 0.5 g. recrystallized benzidine in 50 ml. absolute alcohol and 0.1 g. sodium nitroprusside in 10 ml. distilled water, diluted to 100 ml. Solution B is a mixture of 50 ml. absolute alcohol, 3 ml. glacial acetic acid, 0.5 ml. 30% hydrogen peroxide, and 0.1 g. sodium nitroprusside, diluted to 100 ml. Freshly prepared solutions must always be used, and benzidine must be purified by recrystallization from alcohol.—*H. P. Riley.*

### PLANT MICROTECHNIC

**ERIKSON, D.** Certain aspects of resistance of plum trees to bacterial invasion of *Prunus* sp. by *Pseudomonas mors-prunorum* Wormald. *Ann. Appl. Biol.*, 32, 112-17. 1945.

Resistant and susceptible varieties of plums were inoculated with *Pseudomonas mors-prunorum* Wormald and a histological study was made of the necrotic areas induced in the stem. Freehand sections of fresh bark from vicinity of inoculation were fixed in formalin-acetic-alcohol and stained with 1:10,000 aqueous methylene blue. In normal tissues, lignin was green, cellulose blue, and pectic substances violet; in diseased areas, tissues were brown except that lignin of the bundles was green. Paraffin sections made by the *n*-butyl alcohol, cedar oil and paraffin technic were stained with safranin and Harris' hematoxylin or fast green, or with methyl violet and erythrosin. The pathogen penetrates the tissues between the cells, causing plasmolysis of cell contents, disintegration of cell walls, and gradual invasion by bacteria. The details of invasion are the same for both varieties but in the resistant one it progresses to a less degree and periderm formation shuts off the invaded tissue at a very early stage.—*H. P. Riley*

**JOHNSON, L. P. V.** A rapid squash technique for stem and root tips. *Canad. J. Res., Sec. C*, 23, 127-30. 1945.

Chromosome studies were made from the growing point of the stem and from root tips by the following technic: fix in Carnoy's fluid, 30 min.; macerate and hydrolyze at room temperature in 1 part concentrated HCl and 1 part 95% ethanol, 5 to 10 min.; rinse in two changes of water as quickly as possible; stain in leuco basic fuchsin, one hour or longer; transfer to drop of 50% acetic acid on slide; cover; squash by tapping with a pencil; apply pressure with finger, protecting cover glass with absorbent paper; allow to stand until drying begins under cover glass, about 10 min.; remove cover glass; flood cover and slide twice with 95% ethanol and three times with *n*-propyl acetate at about half-minute intervals; place drop of Canada-balsam-xylene on slide; replace cover glass. The leuco basic fuchsin is prepared as follows: dissolve 1 g. basic fuchsin by pouring 200 ml. boiling distilled water over it; shake thoroughly; cool to 50° C.; filter; add to filtrate 2 g. potassium metabisulphite and 20 ml. *N* HCl; shake; stopper; bleach in the dark, 24 hr.; add 0.5 g. decolorizing carbon; shake one minute; filter through coarse filter paper. The new feature of this method is the use of *n*-propyl acetate as a dehydrating agent because it is far less toxic than is 1,4-dioxane. It was especially good for species of *Abies*, *Picea*, *Pinus*, *Pseudotsuga*, *Thuja*, and *Tsuga*. For tender material, as tomato root tips, it was preferable to reduce the strength of the macerating agent to equal proportions of 95% ethanol and 25% HCl.—*H. P. Riley.*

## MICROÖRGANISMS

**CROSS, JOY BARNES.** A rapid fixation technique for Trematoda and Hirudinea. *Texas Rept. Biol. and Med.*, 3, 101-4. 1945.

A method is described by which ninety well flattened liver flukes may be prepared in an hour, which is considerably faster than the usual procedure of tying the animals between glass slides. Rapidity is particularly important because the period of survival after the host's death is short and only live specimens can be used.

The procedure used is as follows: Tilt a dark-bottomed dissecting pan containing a small amount of SUSA fixative in such a way that the upper two-thirds of the pan remains dry. Place a strip of toilet paper in the dry portion, lay a fluke on one end, and brush flat with a camel's hair brush. Fold the opposite end of the paper over the animal, brush a few times, and cover with a piece of glass leaving some of the paper exposed. While pressing the brush handle against the glass with one hand, use the other to grab the exposed paper with paraffin-tipped forceps and to pull the preparation downward into the fixing solution. The weight of the glass is sufficient to maintain the flattened state while other flukes are prepared in the same way. When the first one has been in the solution long enough to become opaque and to retain its flattened condition, remove the glass and surrounding paper and transfer to a larger container of fixing solution for approximately 12 hours.

With this method, fixation is achieved superficially in 2 or 3 minutes whereas the older method requires 4 hours. The shorter time is especially desirable because it is generally recognized that rapid fixation minimizes the production of artifacts. This method can also be used for leeches and probably for other Trematodes.—*Jean E. Conn.*

**SAMUEL, G., and GARRETT, S. D.** The infected root-hair count for estimating the activity of *Plasmodiophora brassicae* Woron. in the soil. *Ann. Appl. Biol.*, 32, 96-101. 1945.

To study the effect of soil conditions upon the incidence of a soil-borne parasite, it is necessary to measure disease intensity in the soil. The intensity of infection of cruciferous seedlings by *Plasmodiophora brassicae* Woron. can be measured by counting the number of infected root hairs on the tap roots of young seedlings by the following technic: inoculate tumblers of soil with a spore suspension; plant 12 presoaked cabbage seeds in each tumbler; cover with 0.25 cm. soil; cover tumblers with glass lids and keep at 25° C. for 1 week; wash out; drop the 5 strongest seedlings into 1% aceto-carmin where roots may be left for several months; place tap roots under long cover glass on microscope slide ruled at 1 mm. intervals; count under 1 in. objective and 10× ocular using mechanical stage the number of root hairs containing zoosporangia along 2 cm. of seedling tap root; infected root hairs are counted on each side of the root only and not on branch rootlets. This method makes it possible to study initial stage of infection with greater quantitative accuracy than by any previous method.—*H. P. Riley.*

**SEMMENS, C. S.** *Pythium de Baryanum*. *Microscope*, 5, 257-64. 1945.

The relation of the fungus to the host can be observed in a portion of the root of an infected cress seedling. The best part to use for this purpose extends for about a centimeter above the point of attachment to the remains of the seed. This is placed on a slide, covered with a drop of cotton blue in lactophenol; after a few minutes the dye solution is replaced with plain lacto-phenol. The hyphae of the fungus are stained deep blue; the host tissues are unstained or only faintly tinged.—*C. E. Allen.*

## HISTOCHEMISTRY

**HAUSER, E. A., and LE BEAU, D. S.** Microscopic studies of lyogels. Ultra-illumination by incident light. *Ind. Eng. Chem.*, 37, 786-9. 1945.

The preparations are made by dispersing or dissolving the colloid to be studied in an appropriate solvent and spreading this solution on a nonmiscible liquid. A piece of fine wire gauze is then coated by being lifted through the solution, and is placed on a microscope slide. The Ultrapak ultramicroscope, which gives

circular illumination of the preparation from above, is used. Diffusion of light caused by reflections from uneven surfaces or from opaque specimens can be avoided, and various micromanipulations under the microscope are possible without blurring of the image.—*R. T. Whittenberger.*

**JOYET-LAVERGNE, P.** La mise en évidence d'une action de la vitamine C sur la cellule vivante, grace a l'intervention de l'adrenaline. *Compt. Rend. Acad. Sci.*, 217, 327. 1943.

A thin section of living tissue is divided into three parts. One part is placed in water, the other two in a drop of adrenaline in a 1 to 1000 dilution. After 8 minutes a drop of water is added to the sections placed in water and to one of the sections placed in adrenaline; a drop of ascorbic acid (1 to 1500) is added to the second adrenaline preparation. After 10 min. a quantity of a leucoderivate of one of the following dyes is added to each of the three preparations: phenosafranin, neutral red, cresyl blue and Nile blue. While the type of changes occurring in the various preparations are not described, the author states that they indicate that adrenaline slightly increases the intracellular oxidation and that adrenaline plus ascorbic acid greatly increases it.—*Elliott Weier.*

**SERRA, J. A.** Improvements in the histochemical arginine reaction and the interpretation of this reaction. *Portugaliae Acta Biol.*, 1, 1-7. 1944.

The author reviews the directions for the histochemical detection of arginine, previously published by himself and Queiroz Lopes, as follows: Fix tissue in alcohol-formalin; or if fixed in some other cytological fixative, harden the fixed tissue 12-24 hr. in 1:3 formalin. Immerse pieces in a mixture of the following: 0.5 ml. of 1%  $\alpha$ -naphthol in 96% alcohol, diluted 1:10 in 40% alcohol just before use; 0.5 ml. *N* NaOH; 40% aqueous urea. After 12-15 min. add 2% sodium hypobromite (prepared by throwing 2 g. liquid bromine into 100 ml. of 5% aqueous NaOH, agitating and cooling), and allow to act for 3 min. Then add 0.2 ml. of the urea solution and 0.2 ml. of the hypobromite solution with constant stirring; after 3-5 min. the color has reached its maximum. Then remove pieces and place for 2-3 min. in pure glycerol, then changed to fresh glycerol. Make cytological preparations by squeezing or smearing as for aceto-carmin. Or, if desired, make permanent preparations by passing the smears or very thin pieces through 96% alcohol into some alcoholic mounting medium such as Euparal.

The resulting red-orange coloration is characteristic of arginine and is given by only a few other rare compounds (glicociamine, galegine, agmatine) that can occur in biological materials. In practice therefore, it is a specific histochemical reaction for protein-arginine. Several photomicrographs are given showing the presence of arginine, demonstrated by this technic, in certain cytological material.—*H. J. Conn.*

**SERRA, J. A.** Sur la composition protéique de chromosomes et la réaction nucléale de Feulgen. *Bol. Soc. Broteriana* (Portugal), 17, 203-16. 1943.

Using histochemical methods, the writer makes an investigation of the nature of chromosomes and of the significance of the Feulgen reaction. He shows that the proteins of the metaphase chromosomes are chiefly of the basic type, having a high percentage of arginine. Tests to show the existence of an acidic protein, chromosomine (claimed by the Stedmans to constitute more than 50% of the chromosome substance) have not been conclusive. At present it is not possible to assert that chromosomine or any other acidic protein is an important constituent of the metaphase chromosomes. The nucleal reaction of Feulgen is perfectly localized, and is a positive reaction to demonstrate the presence of thymonucleic acid, *in situ*. The staining with fuchsin after treatment with nucleases proves that the nucleal reaction is not due to an assumption of color by an acidic protein.—*H. J. Conn.*



# STAIN TECHNOLOGY

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## PROGRESS IN THE STANDARDIZATION OF STAINS

### RECENT ADVANCES

In recent editorials of this series (during 1944 and 1945), hints have been made to the effect that plans were under way which the Stain Commission hoped to bring to fruition after the war. Some of these plans represented projects already well started while others were still for the future. It seems well now to try to size up the situation and to see how well the plans are maturing.

### PUBLICATIONS

The fifth edition of Biological Stains, which has been requiring considerable work for over a year, and which has been delayed time and time again by difficulties with which the printing industry is faced these days, is now on the press; it is hoped that its publication will not be very much behind that of this issue of STAIN TECHNOLOGY. It is unfortunate that the fourth edition should have gone out of print during the war, at a time when stains were in such demand; but that could not be prevented, and the very situation which has caused the demands for stains has also caused the delays in getting any kind of work (e.g. printing) done on schedule. It is hoped, however, that the fifth edition will be enough better than the fourth to make up for the delay.

Staining Procedures is now complete, with the publication of its tenth leaflet (an index) within the last few months. Although this loose-leaf manual is not so well known as the Commission's older publications, there has been considerable demand for it, in fact a rather surprising demand, considering the small amount of advertising that has been given the publication.

A 20-year index to Stain Technology has been prepared, which should be ready for publication in a month or two. This cannot be promised for any definite time, as the typesetting is not yet complete and no date for its going to press has yet been set. Previously it has been the custom to print a 5-year index at the end of every fifth volume and to include it with the first issue of the following volume. The 5-year index has been omitted after volume 20, because of the contemplated 20-year index; the latter will be too

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long to include as part of a regular issue. It will have to be sold as a separate publication.

#### NEW ASSAY METHODS

The testing of samples submitted for certification has always been of three sorts: tests for actual performance; chemical assay to determine dye strength; spectrophotometric tests to learn whether or not the sample is true to type.

The tests for actual performance are being improved all the time in little particulars; at their best, however, the tests selected do not represent an ideal method of assay, and it is hoped that some day chemical and spectrophotometric methods will replace them. Until such time, however, they must continue to be employed; in fact, it is necessary to lay more stress on them than on chemical methods, which do not always correlate with performance. Imperfect as they are, however, they were regarded as of sufficient significance to be accepted by Army and Navy, during the war, as a basis for the approval of stains for the armed forces. The methods now in use are to be found in an appendix of the fifth edition of *Biological Stains*.

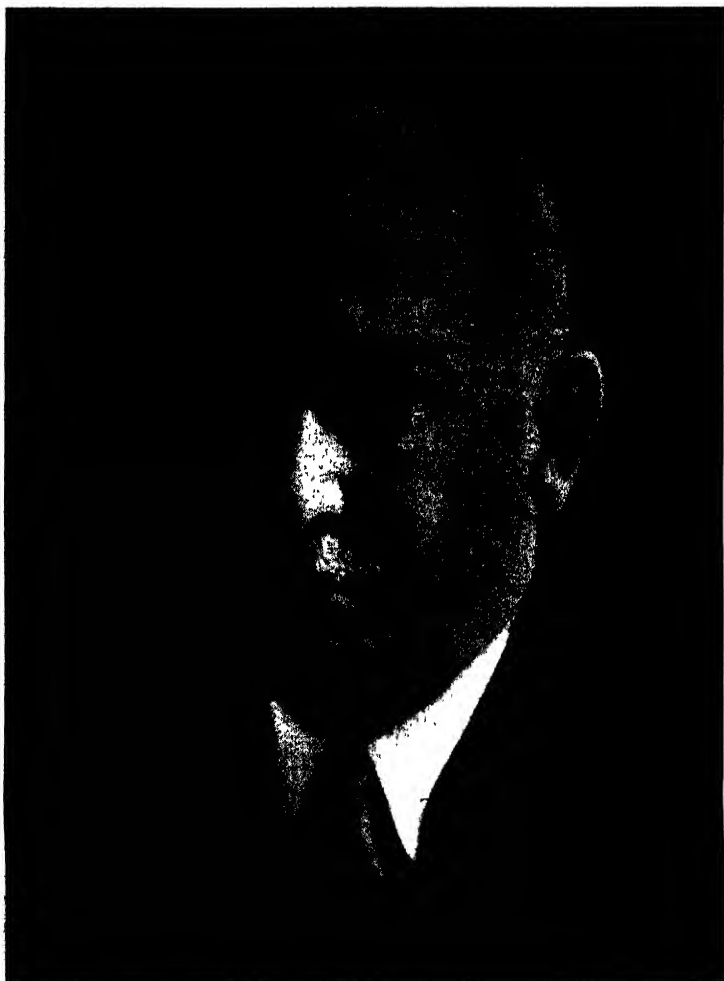
Chemical assay methods, primarily titration with titanium chloride to indicate dye strength, are also being perfected, little by little. They have been drawn up now in sufficiently definite form so that it has proved possible to transfer the testing from one laboratory to another without experiencing serious difficulty. These methods also are included in the appendix of *Biological Stains*.

Spectrophotometric methods are not so well standardized. They are given in the same appendix; but the methods there given are subject to revision at the present time due to the availability of new types of spectrophotometers more precise than the older instruments. Fortunately arrangements have been made to review these methods at Geneva, and it is expected that in a short time, distinctly improved procedures for spectrophotometric assay will be available. The first of a series of papers on this subject is given in this issue of *STAIN TECHNOLOGY* (p. 49).

#### RESEARCH WORK

The Stain Commission has begun a new departure in making a grant to Northwestern University for the continuance of research work in neurological methods formerly carried on by Dr. H. A. Davenport, but which was interrupted by his service in the war. Readers of *STAIN TECHNOLOGY* will recall his papers that have appeared from time to time in these pages. It is gratifying that the Stain Commission is now in a position to enable him to continue the work again.—H. J. CONN.





**CLARENCE ERWIN McCLUNG**  
**1870-1946**

## CLARENCE ERWIN McCLUNG

### In Memoriam

In the sudden death of Clarence Erwin McClung on January 17, 1946, the field of microtechnic lost a distinguished devotee.

C. E. McClung was born in Clayton, California, April 5, 1870. His father was a civil and mining engineer and his mother the daughter of a physician. He grew up in the state of Kansas where he received his education and embarked upon his professional career.

As a boy he displayed a marked interest in chemistry, and after working in his uncle's drugstore, enrolled in the School of Pharmacy at the University of Kansas, receiving the Ph. G. degree in 1892. After teaching chemistry and pharmacy for a year, he entered the college and continued in graduate work, receiving the A. B. degree in 1896, the A. M. in 1898 and the Ph. D. in 1902.

Dr. McClung early developed a dominating interest in the microscope and in microscopical technic, and he entered Professor S. W. Williston's class in animal histology. Here he displayed so much ability and interest that, half-way through the course, Dr. Williston asked young McClung to take charge. In this way he became embarked upon a career in zoology. His work on chromosomes brought him world renown. His discovery of the chromosomal mechanism of sex-determination in insects was a landmark in the development of an understanding of the relations between chromosomes and body characteristics.

From the beginning of his career Dr. McClung was interested in the microscope and the technics employed in its use. As early as 1892 he published a paper on glycerin-mounting and a year later one on "The use of stains in microscopy". Other publications on technic appeared from time to time culminating in the "Handbook of Microscopical Technique" which he prepared with the help of a group of collaborators. First published in 1929, it was followed by a second enlarged edition in 1937. Dr. McClung was mechanically inclined and he devised improvements in microscope design, so that eventually a "McClung Model" was produced by one of the microscope manufacturing companies. When Dr. McClung became director of the zoological laboratory at the University of Pennsylvania in 1912, he took charge of the course in microscopical technic and directed it until his retirement in 1940. In this course he stressed the fundamental principles upon which the technical methods are based.

Dr. McClung's keen intellect, genial personality and administrative ability brought him many responsibilities both inside and outside the universities which he served. At the University of Kansas he was

made head of the department of zoology in 1901, only five years after receiving the A. B. degree, and served on many administrative committees. At the University of Pennsylvania he interested himself in curricular reforms and in improvements in facilities and opportunities for graduate study and research. Here also he was chairman or member of many important committees. At the Marine Biological Laboratory, Woods Hole, Mass., where he and his family spent most of their summers after 1912, he became trustee in 1913, member of the investigative staff in 1914, and served on such important committees as that to secure endowment, the committee on plans for the new permanent buildings, the library committee and the executive committee.

In 1917 Dr. McClung was made chairman of a zoology committee in the Division of Biology and Medicine of the National Research Council. In 1919 he became the first chairman of the new Division of Biology and Agriculture which he helped to organize. While in this position he initiated plans for a comprehensive abstracting service in the field of biology and for methods of improving and standardizing biological stains. Out of these efforts arose the formation of the Union of American Biological Societies of which he was president from 1922 to 1930. This organization, with others, sponsored Biological Abstracts, and he was president of its Board of Trustees from 1925 to 1938. He was also editor of its section on animal cytology from the beginning.

In regard to the project to improve and standardize biological stains, Dr. H. J. Conn, President of the Biological Stain Commission, has this to say, in correspondence:

"It was about this time that my own interest in improving and standardizing American biological stains began. These investigations were begun in 1920 or 1921 and were at that time confined purely to the bacteriological uses of those few stains which bacteriologists used in quantity. At the same time Dr. McClung, unknown to me, was becoming interested in the same problem from the cytological angle. The stain which had given him the most trouble was hematoxylin and his method of attacking the problem was by attempting the laboratory manufacture of this dye and in this way learning what was really at fault in the American commercial product. This was obviously an entirely different method of attack from that which I was pursuing from the bacteriological angle—namely, the coöperative investigation of a considerable series of commercial samples to learn which were the more satisfactory.

"As Director of one of the divisions of the National Research Council, Dr. McClung was naturally interested in seeing the problem attacked

on a wider basis as a coöperative project under Research Council auspices. When he learned that bacteriological investigations on the subject were already under way, that equally difficult problems were being faced in other biological lines, and finally, that certain commercial men were just as eager to see the subject standardized, it seemed quite an obvious move to call a conference of all those interested in the subject. He may have been the first one to propose this conference, although before the step was actually taken, his term of office in the National Research Council was over, and his successor, Dr. L. R. Jones, called the conference. Certainly Dr. McClung was at the meeting, partly as the retired Director of the Division and partly as a representative of zoological science, and he took a very active part in the discussion. The result of this conference need not be detailed here, except to say that the Biological Stain Commission was the eventual outgrowth of it."

As a man, Dr. McClung was known not only for his keen analytical mind, lively imagination and active sense of humor, but also for his natural simplicity and sympathetic friendliness. These qualities made him a delightful companion on almost any occasion. He was always ready to offer kindly counsel to the many associates and students who sought his advice, and his unselfish concern with the interests and welfare of those around him was proverbial.



AT WOODS HOLE

The artistic side of his nature revealed itself in a liking for composition and literature, developed as an undergraduate; in his devotion to various forms of music and pictorial art; in his mastery of different methods of drawing as revealed by the expertly executed illustrations in his scientific papers; in his enthusiasm for photography, and an abiding interest in dramatics.

As a teacher, Dr. McClung sought to develop the innate capabilities of the individual. Even in the elementary course in zoology, which he taught at Kansas, he introduced "unknowns" to arouse the investigative interest which most young people have but which is all too often prevented from being exercised. By personal interviews with his students, he tried to learn the reasons for varying degrees of

success or failure and to stimulate efforts toward improvement. As editor of the *Journal of Morphology* from 1920 until his death, he labored to help zoologists, especially the younger ones, to an appreciation of the best methods of writing scientific papers.

Dr. McClung's abilities and accomplishments were recognized in many ways. He was president of the Central Branch of the American Society of Zoologists, 1910; president of the combined Society, 1914; president of the national society of Sigma Xi, 1917-21; vice-president of Section F (Zoology), A. A. A. S., 1926; president of the American Society of Naturalists, 1927; president of Beta Beta Beta Honorary Biological Fraternity, 1936 until his death, and was a member of the American Association of Anatomists, American Philosophical Society, the Kansas Academy of Sciences, the Washington Academy of Sciences, and National Academy of Sciences. In 1922, during the scientific meetings in Boston, a testimonial dinner was tendered to Dr. McClung, celebrating his 25 years as a teacher of zoology. In 1930 he represented the U. S. Government, the American Philosophical Society and the National Academy of Science at the International Congress of Biology at Montevideo. In 1933-34 he was visiting Professor at Keio University, Tokyo, Japan.

At the time of his retirement in 1940 Dr. McClung was made Emeritus Professor and awarded the honorary degree of Doctor of Science at the Bicentennial Commencement of the University of Pennsylvania. At a dinner held in his honor he was presented with a dedicatory volume of the *Journal of Morphology* to which 30 of his former students contributed papers; a "McClung Model" research microscope; and a bound volume containing more than 200 letters of greeting from friends throughout the world.

The year following his retirement was spent at the University of Illinois as acting head of the department of zoology. In 1941 the University of Kansas initiated the awarding of Distinguished Service citations and Dr. McClung was among the first group of alumni to be thus honored. In 1942 he was given the honorary degree of Doctor of Science by Franklin and Marshall College. In 1943, when Dr. Laurence Irving entered military service, Dr. McClung was invited to become acting head of the department of biology at Swarthmore College.

Dr. McClung's friends throughout the world now mourn the passing of a distinguished scientist, a great teacher and a true friend.

D. H. WENRICH, *Philadelphia, Pa.*

## COMPARATIVE ABSORPTION READINGS OBTAINED WITH SPECTROPHOTOMETERS OF VARIOUS TYPES\*

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With the technical assistance of MARY A. DARROW.

One of the most important means of identifying a dye is by spectrophotometry. This method has been used as a criterion for judging the samples of stains submitted by manufacturers ever since the certification plan for biological stains was adopted. All specifications that have been drawn up for biological stains, such as those given in the 7th edition of the National Formulary, have included spectrophotometric requirements.

The principle of the method has been discussed in so many other places that it does not need to be repeated here. It is enough to explain that by means of the spectrophotometer, an absorption curve can be obtained which is characteristic for any dye and is seldom displayed by any other dye or mixture of any two dyes. The important points in regard to any such curve are the following: whether it shows just one or more than one maximum; the wave length ( $\lambda$ ) at the point of greatest absorption; the width and shape of the absorption band, as indicated by the slope of the curve on each side of the maximum.

The initial work by the Stain Commission on the spectrophotometry of biological stains was performed with a visual type spectrophotometer of Bausch and Lomb manufacture, and until recently this instrument was used as a means of obtaining all standard values to which later analysis could be referred. As a matter of fact, when this work was begun (some 20 years ago), no other type of spectrophotometer was available.

Within recent years, photo-electric spectrophotometers have been perfected and are becoming very popular. They have several advantages: greater speed and precision; and freedom from personal error due to eye fatigue or to varied sensitivity of the eye to light in different parts of the spectrum. The simplest form of photo-electric instrument calls for visual reading of the dials; this requires some time, but much less than when the comparison of color intensity must also be made visually. Among the speediest of modern

\*Journal paper 669, New York State Agricultural Experiment Station, Geneva, New York, March 6, 1946.

spectrophotometers is the General Electric recording instrument which automatically draws a graph showing the transmittance throughout the entire spectrum; a complete curve for any sample can be made in a very few minutes.

These different types of spectrophotometers differ from one another in another important respect: namely, the width of the slit through which the light from the prism is allowed to pass to the photometer.

TABLE 1. COMPARATIVE SPECTROPHOTOMETRIC DATA OBTAINED ON THE VISUAL (B & L) VS. THE PHOTOELECTRIC (BECKMAN) SPECTROPHOTOMETER.

Dye	Nat. Formulary standards		Comparative determinations			
	Wave lengths of points selected for ratio	Ratio tolerance	Cert. No. of sample tested	Absorption maximum ( $\lambda$ )	B & L Ratio	Beckman Ratio
Yellow Dyes, $\lambda = 435-445 \text{ m}\mu$						
Auramin O*	470/490		NAu-1	438	7.50	7.95
Martius yellow*	500/530		NYM-8	445	21.3	
Brown and Orange Dyes, $\lambda = 465-490 \text{ m}\mu$						
Bismarck brown Y*	500/530	1.75-2.28	NN-11	470	1.77	1.94
Methyl orange*	500/530	0.96-0.98	NM-9	465	0.96	1.59
Orange II*	500/530	1.95-2.01	COB-2	490	2.01	1.96
Orange G*	500/530	4.40-5.80	NO-10	480,495	5.20	5.75
Red Dyes, $\lambda = 510-560 \text{ m}\mu$						
Sudan III	500/530	1.02-1.13	NY-8	510	1.07	1.07
Congo red	500/530	1.19-1.21	NQ-12	510	1.19	1.17
Sudan IV	500/530	0.90-0.94	NZ-14	520	0.92	0.92
Eosin Y	505/535	0.82-1.01	LE-18	525	0.96	0.58
Eosin B	515/545		NEB-10	525	1.66	1.36
Erythrosin B	515/545	1.04-1.55	NER-8	525	1.09	1.32
Erie garnet B	500/530		NEg-1	525	0.94	0.88
Ethyl eosin	515/545	0.85-1.02	NEe-3	535	0.89	0.57
Safranin O	515/545	0.98-1.08	NS-19	535	1.00	0.87
Neutral red	515/545	0.73-0.77	NX-12	ca. 545	0.75	0.72
Rose Bengal	540/570		CRb-4	555	1.98	1.26
Phloxine B	530/560	0.95-1.50	NPh-11	550	1.07	0.70
Pararosanilin	530/560	1.12-1.17	NF-48	548	1.17	0.96
Fuchsin, acid	530/560		NR-18	552	0.95	0.81
Rosanilin	530/560	0.87-0.91	CF-30	552	0.89	0.79
Pyronin Y	530/560	0.76-1.00	NP-10	552	0.87	0.65
Pyronin B	530/560	0.50-0.60	NP-11	558	0.51	0.41
Alizarin red S	545/575		NAR-6	558	1.16	1.10

\*These dyes have their absorption maxima so far into the violet light that it proved impractical, with the visual instrument, to record ratios between points on either side of the maximum, and the two points selected were both therefore on the descending leg of the curve. With the Beckman instrument it is possible to get good readings in the violet part of the spectrum; so it is possible to select new points for the ratios of these dyes, one of which is on either side of the maximum; see Table 2.

TABLE 1. *Concluded.*

Dye	Nat. Formulary standards		Comparative determinations			
	Wave lengths of points selected for ratio	Ratio tolerance	Cert. No. of sample tested	Absorption maximum ( $\lambda$ )	B & L Ratio	Beckman Ratio
Violet and Blue Dyes $\lambda=585-670\text{ m}\mu$						
Methyl violet 2B	575/605	1.30-1.50	NMv-9	588	1.44	1.23
Crystal violet	575/605	1.02-1.10	LC-20	595	1.06	0.92
Thionin	590/620	1.40-1.55	NT-13	608	1.53	1.05
Anilin blue WS	590/620	0.95-1.05	NK-6	610	0.95	0.95
Indigo carmine	590/620	0.78-0.82	NI-8	615	0.81	0.79
Azure C	605/635	1.00-1.12	NAc-3	625	1.11	0.87
Cresyl violet	620/650	1.14-1.21	NW-15	630	1.18	1.08
Brill. cresyl blue	605/635	0.82-0.95	NV-32	632	0.88	0.86
Azure A	620/650		NAz-11	635	1.23	1.05
Toluidine blue O	620/650	0.99-1.39	NU-3	640	0.99	0.87
Nile blue A	620/650	0.69-1.04	NNb-3	640	1.00	0.89
Azure B	635/665		NAb-2	660	0.92	0.81
Methylene blue	635/665	0.56-0.62	LA-13	668	0.61	0.54
Green Dyes $\lambda=620-640\text{ m}\mu$ (400-430 $\text{m}\mu$ )						
Fast green FCF	605/635	0.76-0.83	NGf-8	628 (430)	0.80	0.57
Malachite green	605/635	0.83-0.88	NMg-12	630 (420)	0.87	0.68
Brilliant green	620/650	1.51-1.62	NBg-15	ca. 635 (420)	1.54	1.16
Methyl green	620/650	0.88-1.00	NG-25	640 (420)	0.95	0.90
Janus green B	635/665	0.95-1.14	NJ-8	ca. 635 (400)	1.11	1.04
Light green SFy	620/650	0.96-1.06	NI-8	640 (430)	1.03	0.76
Black Dyes						
Chlorazol black E	590/620		NBc-1	605 (480)	1.05	1.05
Nigrosin	575/605	1.00-1.13	NNi-6	580, 615	1.01	0.99

The narrower the slit, the more nearly monochromatic the beam of light which can be measured. It is obvious that the narrower the slit, the more definitive are the wavelength readings that can be made; but it is equally clear that with the visual type instrument, the sensitivity of the human eye sets a limit to the narrowness which is practical. It proves possible therefore to use a narrower beam of light with the photo-electric instrument than with the visual instrument. With the Beckman spectrophotometer, for instance, a nominal wave length band width of 0.6 to 3.2  $\text{m}\mu$  can be used in the visual spectrum, the smallest size being selected for each wavelength reading with which the required sensitivity of the phototube can be obtained. With the Bausch and Lomb visual instrument employed in the work here referred to, the band width that proved practical was similar,

but could not be accurately controlled. With the General Electric Recording Spectrophotometer, it may be adjusted automatically for a uniform band width, ordinarily 10  $m\mu$ , although good results can be obtained at 8 $m\mu$ .

When spectrophotometric study of stains was begun the effort was made at first to determine the exact location of the absorption maximum for each sample. With the visual type of instrument then in use, this proved a time-consuming operation and a short-cut was soon devised. It was found that if the extinction coefficient ( $E$ ) of two points were obtained, one on either side of the maximum, the ratio between these two values of  $E$  can be used as a measure of the point of maximum absorption. This method was put into practice because of the ease with which the determinations can be made; and, in drawing up the specifications for stains which are now included in the National Formulary, the limits of tolerance are given for this ratio in the case of each dye.

All these ratios, as previously explained, were computed with the use of the visual type of instrument of Bausch and Lomb manufacture. They proved to check very closely with those obtained using the G. E. Recording Spectrophotometer, but not always with those secured with the Beckman Spectrophotometer which is a non-recording photo-electric instrument. The difference in the results has not yet been explained.

Whatever the explanation of the differences, it has seemed desirable to make a series of graphs for typical samples of all the certified biological stains on a Beckman instrument, if for no other reason than because of the increasing popularity of spectrophotometers of that type. The results thus obtained, in comparison with those secured with the visual type instrument, are proving quite interesting and it is planned to present them in a series of papers. The data so far secured are presented in Tables 1 and 2. Table 1 shows: (1) the National Formulary standards, which indicate the limits of ratios as found in the past, with the visual type of instrument, for satisfactory samples of the various dyes; (2) the ratio found for one particular satisfactory (and typical) sample of each dye, using the visual spectrophotometer; (3) the ratio now found for the same sample of each dye, using the Beckman instrument. It will be seen that in some instances the latter ratio is nearly the same as obtained with the visual type of instrument, but most often it is distinctly different; frequently, it lies entirely outside the tolerance of the old standards. It is clear that if such ratios are still to be used, but the data obtained with the

photo-electric type of instrument, entirely new standards must be adopted for many of the dyes.

In some instances, the points selected in the past for obtaining the absorption ratio were chosen wholly with regard to the limitations of the visual instrument, and it proves that with the photo-electric instrument a more significant ratio can be obtained if the points are located at a slightly different part of the spectrum. This is true particularly in the case of yellow, brown, and orange dyes, as their primary absorption is in, or near, the violet, where accurate visual readings are difficult. Because of this difficulty in recording readings in the violet, the two points formerly selected in the case of brown, orange and yellow dyes were both on the descending leg of the

TABLE 2. RECOMMENDED RATIOS\* FOR DYES WITH THEIR ABSORPTION MAXIMA IN THE VIOLET PART OF THE SPECTRUM.

	Wave-lengths of points selected		Observations recorded on Beckman instrument	
	Nat. Formulary standard	New recommendation	Cert. No. of sample tested	Ratio at points of new recommendation
	m $\mu$	m $\mu$		
Auramine O	470/490	425/455	NAu-1	1.24
Bismarck brown Y	500/530	460/490	NN-11	1.10
Martius yellow	500/520	425/455	NYm-3	0.98
Methyl orange	500/530	425/455	NM-9	1.01
Orange G	500/530	470/510	NO-10	1.03
Orange II	500/530	4 0/510	COh-2	0.92

\*Although these new ratios are recommended to replace the older ones, it must be recognized that all such ratios may be supplanted in the future by other types of spectral analyses. (See text).

absorption curve instead of one on each side of the maximum as with the dyes of other colors. As photo-electric cells are quite sensitive to violet light, this limitation does not hold with the Beckman spectrophotometer, and it is possible to select points on each side of the maximum, even with a yellow dye, and thus to secure much more significant ratios. The new recommendations for such dyes are given in Table 2.

As a matter of fact, however, it is entirely probable that with the photo-electric spectrophotometers now available, the short-cut involved in the use of these ratios will no longer be necessary. It is possible to find the actual maximum of the curve so much more rapidly on such an instrument than on the older type that it can be

obtained even in the routine examinations of stains, and values of more significance than the absorption ratio can be readily secured. It is planned to give such values for the various stains in the future papers of this series.

Since completing the above article, the authors have had their attention called to the fact that the majority of the dyes in which they found great discrepancy between the ratios as obtained with the two spectrophotometers were either fluorescent dyes or meta-chromatic stains. Further investigation is needed to show what connection, if any, there is between this dichroism and the discrepancy in the ratios.

## THE DIFFERENTIATION OF PLACOID, CTENOID AND CYCLOID SCALES BY MEANS OF ALIZARIN RED S

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**ABSTRACT.**—This technic has been used successfully by the author for staining, *in situ*, the placoid scales of selachians and the smaller forms of cycloid and ctenoid scales of teleosts. Sections of skin are dissected from the ventrum of the specimen, cleaned of fascia and muscle tissue and fixed in a 10% formalin solution. The section is macerated in several changes of a 3% KOH solution until translucent. Staining is accomplished in a fresh 2% KOH solution to which is added a saturated alcoholic solution of alizarin red S. The section remains in the stain for 24 hours. If necessary, the tissue may be quickly destained in acid alcohol (1% H<sub>2</sub>SO<sub>4</sub> in 95% alcohol). The skin is dehydrated in cellosolve or the alcohol series and cleared in methyl salicylate. Placoid and teleost scales prepared in this manner are stained a brilliant red. The various parts of the denticle are well differentiated.

### INTRODUCTION

The placoid scale or dermal denticle of the selachians is a particularly interesting structure to the comparative anatomist. The concept that this type of scale gave origin, in some manner, to the much modified mammalian tooth seems to be generally accepted today. Though a study of the placoid scale of such forms as *Squalus acanthias* or *Squalus sucklii* is invariably included in a course of general comparative anatomy, the author feels that there has been no satisfactory method evolved for demonstrating this significant structure. The technic usually employed in preparing scales of this type for class use is to corrode the skin in strong (30%) KOH or NaOH so as to completely macerate the soft tissues, allowing the scales to precipitate to the bottom of the container. The scales are then washed, run up through a clearing agent and mounted in balsam. This technic is one recommended by Galigher<sup>1</sup>. Another still less satisfactory method in common use is to clear a small section of skin in glycerin, mounting it finally in glycerin jelly. Neither of these preparations will satisfactorily differentiate the various parts of the placoid scale nor show the

<sup>1</sup>Galigher, A. E. 1934. *The Essentials of Practical Microtechnique*. Albert E. Galigher, Inc. Laboratory of Microtechnique, Berkeley, Cal. See p. 107.

relation of the basal plate to the corium in which it is embedded. The method presented in this paper, primarily for the placoid scale, is also applicable to the small forms of the teleost, ctenoid and cycloid types. The dye used is specific for  $\text{CaCO}_3$  and has been employed by the author<sup>2</sup> and other workers for the staining of embryonic and adult bone.

### TECHNIC

1. *Selection and fixation.* The whole specimen is fixed in 10% formalin for at least 1 week. A large section of the integument is then cut away which later may be trimmed to sizes convenient for mounting. It is best to remove the integument from the ventrum of the specimen where it is comparatively free of concentrations of pigment. All shreds of muscle tissue and superficial fascia are carefully removed from the resected skin.

2. *Maceration.* The skin is macerated in several changes of a 3% solution of KOH for about 72 hours, the length of time varying with the thickness of the tissue. At the end of maceration the skin should appear translucent and white. The scales should remain firmly adherent to the skin and show no evidence of sloughing off. This process may be facilitated by exposure to sunlight or ultra-violet rays.

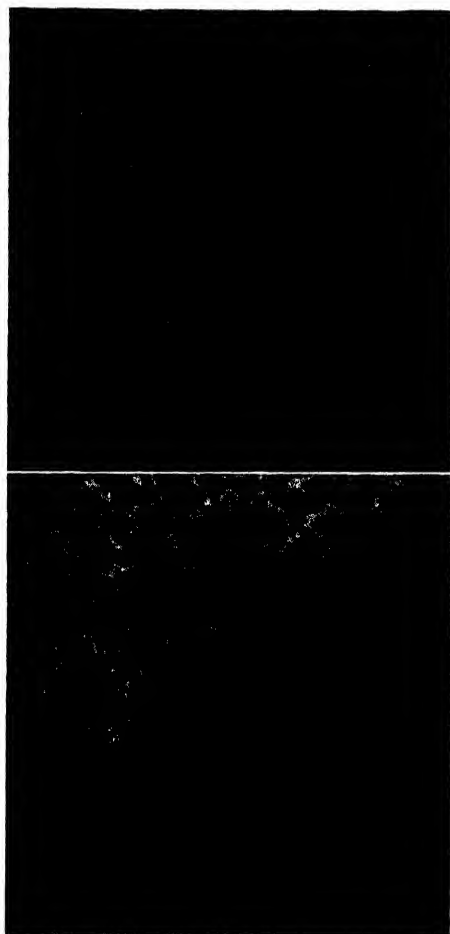
3. *Staining.* The macerated skin is next transferred to a fresh solution of 2% KOH to which is added, by drops, a saturated alcoholic solution of alizarin red S<sup>3</sup>. Enough of the latter is added to the hydroxide solution to color it a deep red. The skin is left in the staining solution for 24 hours, at the end of which time, if maceration has been sufficient, the scales will be stained intensely, the soft tissues remaining unstained. Any excess stain may be removed quickly by placing the tissue in acid alcohol (1%  $\text{H}_2\text{SO}_4$  in 95% alcohol).

4. *Dehydration, clearing and mounting.* Dehydration is accomplished by running the tissue through 2 changes of cellosolve<sup>4</sup> of 2 hours each. Those who prefer the alcohol series may substitute with changes of 50%, 80% and 95% alcohol followed by 2 changes of benzene. Though clearing may be accomplished in any of the standard clearing reagents, the author recommends methyl salicylate (synthetic oil of wintergreen). After dehydration the tissue is transferred to solutions of 25%, 50% and 75% of methyl salicylate in cellosolve, for 2 hours each. It then may be placed in pure methyl salicylate for permanent storage. Small sections of the stained and

<sup>2</sup>Williams, T. W. 1941. Alizarin red S and toluidine blue for differentiating adult or embryonic bone and cartilage. *Stain Techn.*, 16, 23-5.

<sup>3</sup>The dye was from Coleman and Bell, No. 40064 No. 4.

<sup>4</sup>'Cellosolve', manufactured by the Carbide and Chemicals Corporation, N. Y.



Photomicrograph of the integument of *Squalus acanthias*. Soft tissues cleared, placoid scales stained. ( $\times 13$ ). This plate is a stereogram and for relationships in three dimensions it may be viewed through a stereoscope.

cleared integument may be mounted on slides in neutral Canada balsam.

The following modification of this technic is suggested for those who might prefer a simplified method for classroom use; it will, however, render the soft tissues more opaque: Omitting step 4, the skin is transferred directly from the KOH-alizarin solution into a series of 50%, 70% and 80% glycerin solutions for 3 hours each. It may then be stored in pure glycerin or mounted in glycerin jelly.

Where possible, it is urged that these preparations be viewed through a binocular dissecting microscope for the added three dimensional effect. The preparation is further enhanced by the use of blue, transmitted light.

#### DISCUSSION

Selachian placoid scales prepared in the manner described above present a strikingly beautiful appearance and are well differentiated. The denticles are stained a brilliant red in contrast to a white background formed by the cleared integument. The basal plate of the scale, formed of cementum and embedded in the corium, stains very deeply. The extra-integumental spine, composed of dentine capped with enamel, stains less intensely. An added advantage is the fact that the scales remain *in situ*, their relations to one another and to the integument remaining intact.

## THE BODIAN TECHNIC AND THE MOSQUITO NERVOUS SYSTEM

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**ABSTRACT.**—Fixation of mosquitoes with Petrunkevitch's paranitro-phenol fluid with 20 or 25% formalin was found to allow use of Bodian activated protargol technic, where other commonly used fixing fluids yielded no success. Gold toning with subsequent oxalic acid reduction was found to be necessary for Culicid nerve tract preparations.

In preparing a series of sections for a study of the nerve tracts of the mosquito cephalic ganglia<sup>1</sup>, some difficulty was encountered in attempting to use the Bodian activated Protargol technic<sup>2</sup>. This method, which had been developed primarily for vertebrate material, was used successfully with the mosquitoes in question (*Culex pipiens* L., *Culex restuans* Theobald) only after certain modifications of the original technic had been made.

### FIXATION

Eleven fixing solutions were tried in an attempt to find a fixative which would permit good preparations with the technic in question. The fixatives used, and their composition, are described in table 1.

Adult mosquitoes fixed in preparations 1 to 6 (cf. table 1) failed to show fiber tracts in the central nervous system after treatment by the Bodian method. Good fiber tract preparations were obtained after use of fixatives 7 to 11. It should be noted that the Petrunkevitch paranitro-phenol fluid containing 10% by volume of formalin was not suitable for demonstrating nerve tracts, while the same fluid containing 20% formalin was quite adequate. Fixatives 9 to 11 allowed too much shrinkage to be acceptable for use despite the staining success. Thus the paranitro-phenol fluid with either 20% or with 25% formalin was used predominantly as the fixing fluid for the preparation of slides for this study.

### DEHYDRATION AND CLEARING

Specimens were dehydrated either with a graded series of alcohols, or with dioxane. Xylene was used as the clearing agent.

<sup>1</sup>Rogoff, W. M. 1944. The anatomy and metamorphosis of the cephalic ganglia of the mosquito. Cornell U. Abstracts of Theses, 1943, 291-3.

<sup>2</sup>Bodian D. 1936. A new method for staining nerve fibers and nerve endings in mounted paraffin sections. Anat. Record, 65, 89-97.

1937. The staining of paraffin sections of nervous tissues with activated protargol. The role of fixatives. Anat. Record, 69, 153-62.

## EMBEDDING AND SECTIONING

Almost all specimens were embedded in a rubber-paraffin mixture ("Tissue-mat", marketed by the Fisher Scientific Company), though a few were placed in standard paraffin. It was found that both media sectioned nicely and allowed only a minimum amount of fragmentation of the chitinous cuticula of the mosquitoes. Sections were cut mostly at  $10\mu$ .

TABLE 1. FIXATIVES AND COMPOSITION

1. Formol-saline	Formalin	10 ml.
	1% salt solution	90 ml.
2. Petrunkevitch's	Water	60 ml.
Sublimate	Absolute alcohol	40 ml.
	Glacial acetic acid	18 ml.
	Nitric acid	2 ml.
	Mercuric chloride to saturation	
3. Bouin's picro-formol	Picric acid (sat. aq.)	75 ml.
	Formalin	25 ml.
	Acetic acid (glacial)	5 ml.
4. Bouin's picro-formol plus turgitol	Turgitol is a commercial detergent	
5. Davenport & Kline modified Höffker's trichloroacetic (at 70° C.) <sup>3</sup>	Trichloroacetic acid	10 grams
	Glacial acetic acid	10 ml.
	Normal butyl alcohol	60 ml.
	Normal propyl alcohol	20 ml.
6. Petrunkevitch's paranitro-phenol with 10% formalin	60% alcohol	100 ml.
	Nitric acid	3 ml.
	Ether	5 ml.
	Cupric nitrate	2 grams
	Paranitro-phenol	5 grams
	Formalin (reagent)	12 ml.
	(Formalin added just before use)	
7. Petrunkevitch's paranitro-phenol 20% formalin		
8. Petrunkevitch's paranitro-phenol 25% formalin		
9. Petrunkevitch's paranitro-phenol 33% formalin		
10. Petrunkevitch's paranitro-phenol 50% formalin		
11. Formol-ether-alcohol	Formalin (reagent)	25 ml.
	Ether	5 ml.
	70% alcohol	70 ml.

<sup>3</sup>Davenport, H., and Kline, C. L. 1938. Experiments with Bodian's method. *Stain Techn.*, 13, 147-58.

## AFFIXING SECTIONS TO SLIDES

Because of the long chemical and incubation treatment, it was necessary to have the glass slides scrupulously clean, before affixing sections, to prevent undue loss. It was found that slides cleaned in the standard  $K_2CrO_4-H_2SO_4$  mixture, rinsed in water, dried, and used immediately were satisfactory for this work. Mayer's albumen mixture was used as the adhesive. The sections were heated just before dissolving out the paraffin prior to staining.

## STAINING

Preparations were stained with Bodian's silver impregnation technic, involving the use of activated protargol. (Protargol, a brand of strong protein silver, i. e. silver albumose, is marketed by the Winthrop Chemical Company). Details of the staining technic follow:

1. *Impregnation.* Sections are placed in a solution of 1% protargol containing 4-6 grams of metallic copper (copper wire, shavings, sheet, etc.) per 100 ml. of solution, and incubated for 24-48 hours at 37° C. It was found desirable to cleanse the copper with concentrated nitric acid before using. The protargol-copper bath may be used only once. Sections are then washed in distilled water.

2. *Reduction.* Sections are placed in a reducing solution (1 gram hydroquinone, 5 grams sodium sulphite, 100 ml. water) and left for 5 to 10 minutes. They are then washed thoroughly in about three changes of distilled water.

3. *Gold toning.* Gold toning was found necessary for Culicid nervous system preparations. This toning is accomplished as follows:

(a) Sections are placed in a solution of 1% gold chloride, containing about three drops of glacial acetic acid per 100 ml. of solution, until they are decolorized (usually 2 to 5 minutes).

(b) Sections are rinsed rapidly in distilled water—one dip is usually sufficient.

(c) Sections are placed in 2% oxalic acid until they have a faint purplish (or blue) tinge—usually 2 to 5 minutes, after which they are washed in distilled water.

4. *Stabilization.* Residual silver salts are removed in a solution of 5% sodium thiosulfate (hypo) for 5 to 10 minutes, after which the sections are washed thoroughly in distilled water, dehydrated, and mounted in damar or balsam.



## IMPROVED FIXATION IN VITALLY STAINED METHYLENE BLUE PREPARATIONS<sup>1</sup>

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**ABSTRACT.**—The present paper reports that ammonium molybdate dissolved in physiological saline for amphibian and mammalian tissues, and in sea water for squid tissues, forms a fixing solution that greatly reduces cellular distortion in vitally stained permanent methylene blue preparations.

Permanent preparations of tissues stained vitally with methylene blue generally show considerable cellular distortion. This phenomenon has been attributed to abrupt steps in dehydration. Rapid dehydration is necessary because methylene blue is soluble in alcohol even after fixation with ammonium molybdate, and the loss of even minute quantities of stain may alter the final picture at critical points (Cole, 1925, pp. 384-5). To reduce the loss of dye and to permit a more leisurely rate of dehydration, the writer used two methods (Cole, 1934, 1936). In the second method, where isotonic saline was used as a solvent for the fixing agent, less distortion occurred than in the first method. Re-examination of a large series of preparations indicated that the nature of the fixing solution might have had something to do with the degree of distortion. The writer long ago noted that distortion was somewhat reduced, in the methylene blue technic, if Ringer's solution was used as a solvent for the ammonium molybdate used in fixation (Cole, 1925, p. 377). Subsequent work has shown that physiological saline is similarly effective for amphibian and mammalian tissues.

Carlton (1922) pointed out that the use of isotonic saline in place of distilled water has no effect when used with mercuric chloride as a fixing agent, but that it did considerably reduce distortion when used with formalin in fixation. Similarly, Stowell (1941) in a study on the effects of various methods of fixation, dehydration, and embedding on tissue volume, noted that the use of isotonic diluents slightly improved formalin fixation.

A long series of studies on the integument of the squid, *Loligo pealii*, Les., has shown that ammonium molybdate dissolved in sea water gives far less cellular distortion than when the salt is dissolved

<sup>1</sup>This investigation was aided by a grant from the Williams 1900 Fund.

in distilled water. Thus it appears that with improper fixing solutions the major part of the distortion occurs previous to the admittedly harsh but necessary dehydration procedure. That the use of sea water instead of distilled water is also applicable to other fixing agents has been shown by Young (1935). Young showed that both Champy's solution and "4% formaldehyde" caused less distortion in the stellate ganglion of *Sepia* when sea water was used in place of distilled water in preparing the respective fixing solutions. Both Carlton (1922) and Young (1935) demonstrated that the effects varied with different fixing solutions. The latter suggested that possibly the use of sea water (and presumably physiological saline) maintained normal osmotic pressures external to the tissues until the slower moving ions of the fixing solution reached the point where they could balance internal pressures. The present paper reports that ammonium molybdate dissolved in physiological saline for amphibian and mammalian tissues, and in sea water for squid tissues, forms a fixing solution that greatly reduces cellular distortion in vitally stained permanent methylene blue preparations.

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## A SIMPLIFIED STAIN FOR HEMOGLOBIN IN TISSUES OR SMEARS USING PATENT BLUE

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With the technical assistance of E. C. THOMPSON, *Assistant  
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**ABSTRACT.**—The following technic, based on the patent blue V hemoglobin reaction, is useful for identifying hemoglobin in tissue fixed in neutral formaldehyde solution and embedded in paraffin:

Stain the deparaffinized, hydrated sections 3 to 5 minutes in the working reagent, prepared by adding 2 ml. of glacial acetic acid and 1 ml. of 3% hydrogen peroxide to 10 ml. of the filtered stock solution (1 g. patent blue, 10 g. zinc powder, and 2 ml. glacial acetic acid). Counterstain 30 to 60 seconds in 1:1000 safranin solution in 1% acetic acid, rinse, dehydrate with alcohols, clear in xylene and mount in clarite. Total time required, 37 minutes.

Blood and tissue and smears may be stained, following fixation in methyl alcohol, by applying the working reagent as above.

The basic principles of this staining technic were described by Lison<sup>1</sup>, who used it in identifying hemoglobin globules and casts in the kidneys of frogs following intraglomerular injections of hemoglobin solutions. In these amphibia it was apparently necessary for him to fix the delicate hemoglobin globules in Slonimski-Lapinski solution (potassium ferricyanide, 2.5–4 g.; 10–20 ml. of 38% formaldehyde solution; 100 ml. water) and make frozen sections of the tissue. Both these procedures offered obstructions to the routine use of the method and we found that both could be eliminated in our work on chemical hemolysis in warm-blooded animals.

We compared the kidneys of mice, rats and guinea pigs containing numerous freshly formed hemoglobin casts and cytoplasmic globules in the epithelium of the convoluted tubules in the following manner: half of one kidney was fixed in ferricyanide formalin and frozen sections cut; the other half was fixed in 4% formaldehyde solution buffered to pH 7.0, embedded in paraffin, and sections cut. The latter is our routine procedure. The staining qualities of the sections prepared by the two methods were compared; leuco patent blue V was used. The results appeared even more satisfactory by following

<sup>1</sup>Lison, L. 1938. Zur Frage der Ausscheidung und Speicherung des Hamoglobins in der Amphibienniere. Beitr. path. Anat. allg. Path., 101, 94–108.

our usual fixation and embedding technic than with the procedure recommended for amphibia. This was not our experience with frogs, in which hemoglobin globules stained very poorly without special fixatives.

Because we had in our files numerous animal tissues<sup>2</sup> stained by hematoxylin-picric-acid technic<sup>3</sup> for hemoglobin, we were able to make a close comparison of the results with these two methods. In all cases the results were satisfactory. The patent blue stain did not give the "false positive" reactions of the other stain which always reacts with trichohyalin granules and, occasionally, with dense keratin. Since the patent blue reaction (peroxidase type) is sensitive, the technic must be precise. For example, in tissue fixed in Zenker's fluid the hemoglobin-staining with patent blue is weak, while if Bouin's fluid is used for fixation of tissue the hemoglobin is not stained.

On the basis of this experience, the simplified patent blue technic is recommended as a good, reliable procedure suitable for routine use under the conditions described.

The simplified patent blue stain technic is as follows:

A. Tissue blocks should not be thicker than 3 to 5 mm. They should be fixed about 24 to 28 hours—prolonged fixation should be avoided—in a 4% formaldehyde solution buffered to pH 7.0. For the best results perfect fixation is necessary. The tissue is then embedded in paraffin and sectioned as usual. We find that sections 5 to 6 $\mu$  thick are the most satisfactory.

B. The patent blue stock solution is prepared as follows:

Patent blue V (CI No. 712, NAC-8137)	1.0 g.
Water, distilled	100.0 ml.
Dissolve the dye in the water, then add:	
Zinc powder, C. P.	10.0 g.
Glacial acetic acid	2.0 ml.

After adding the zinc and acetic acid to the dye solution, bring the mixture to a boil. In a short time a complete decolorization (loss of blue color) will occur. This solution is said to be stable.

To prepare the working reagent:

Filter, just before use, 10 ml. of the stock solution and add 2 ml. glacial acetic acid and 1 ml. commercial hydrogen peroxide (3%).

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<sup>2</sup>From the studies on chemical hemolysis of Dr. Stewart Webster, Biochemist, Industrial Hygiene Research Laboratory, National Institute of Health.

<sup>3</sup>Dunn, R. C., and Thompson E. C. 1945. A new hemoglobin stain for histologic use: a slightly modified Van Gieson stain. Arch Path., 39, 49-50.

**C. To prepare carmine alum:**

Carmine (alum lake)	1.0 g.
Water, distilled	200.0 ml.
Salicylic acid	0.2 g.

Dissolve the dye in the water with the aid of heat; when cool, add salicylic acid as a preservative and filter.

**D. To stain the sections:**

1. Bring paraffin sections to water.
2. Stain in the working reagent (freshly prepared), 3 to 5 minutes.
3. Wash briefly in water.
4. Counterstain in carmine alum, 30 to 60 seconds (or use a weak solution of safranin: 0.1 g. safranin, 1 ml. glacial acetic acid, 99 ml. distilled water.)
5. Dehydrate with alcohols, clear in xylene and mount in clarite.

**E. Results:** Hemoglobin stains dark blue-green, the background a light pink color.

Methyl-alcohol-fixed blood and tissue smears may be stained by applying the working reagent as above. Since the patent blue stain is essentially a peroxidase type of reaction, the granules in certain leucocytes, particularly eosinophils, stain dark blue.



# A SIMPLIFIED METHOD OF PREPARATION OF DI-AMMINE-SILVER HYDROXIDE FOR RETICULUM IMPREGNATION; COMMENTS ON THE NATURE OF THE SO-CALLED SENSITIZATION BEFORE IMPREGNATION<sup>1</sup>

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**ABSTRACT.**—A satisfactory di-ammine-silver hydroxide solution may be repeatedly and consistently prepared by adding 9 or 10 volumes of 10% silver nitrate solution to 1 volume of 28% ammonia water, running in the first 6 or 7 volumes rapidly and proceeding cautiously from then on, shaking until clear after each addition, until a faint permanent turbidity is reached.

The essential nature of Gomori's iron alum treatment and of Wilder's uranyl nitrate step following the Weigert permanganate-oxalic-acid sequence appears to be an oxidation, since the same results may be achieved with chromic acid, hydrogen peroxide, sodium iodate and elemental iodine, and since this step is better omitted on previously chromated material.

The purpose of this paper is to introduce a variation in the manufacture of the ammoniacal silver oxide solution which, by giving a more definite end-point, makes easier the duplication of the solution. Further exploration was made also of the "sensitizing" bath with uranyl nitrate suggested by Wilder and the bath with ferric ammonium alum suggested by Gomori.

According to the Bielschowski-Maresch technic about 2/3 of the 1.08 g.  $\text{AgNO}_3$  in 10 ml. of 10% silver nitrate solution is precipitated by addition of 5 drops or about 0.3 ml. of 40% sodium hydroxide solution (equivalent to about 170 mg.  $\text{NaOH}$ ). Then 28% ammonia water is added drop by drop until the precipitate is almost all redissolved. In practice this exact point is difficult to duplicate, and one or two drops of ammonia water seem to make a considerable difference in the action of the solution on tissues,

This procedure is essentially similar in various Bielschowski variants, and the same general method is followed in the del Rio Hortega diammine-silver carbonate variants. Gomori alone has proposed adding more silver nitrate after the silver oxide has been dissolved. He directed adding further silver nitrate until the brown precipitate

<sup>1</sup>From the Pathology Laboratory of the National Institute of Health, Bethesda, Md.

formed dissolves easily on shaking. In practice this is found to be an extremely vague end-point, and Gomori writes me that his actual practice is to continue the addition of silver nitrate until the precipitate dissolves only with difficulty, or even to the point of a faint permanent turbidity, when he then adds 1 more drop of 2% ammonia water to clear.

Being unimpressed with the importance of having potassium or sodium nitrate as well as, or in place of, ammonium nitrate in the final solution, I tried the procedure of placing a measured quantity of ammonia water in a beaker and adding to it 10% silver nitrate solution to the point of faint permanent turbidity. The amounts consumed corresponded quite closely to the reaction  $\text{AgNO}_3 + 3\text{NH}_4\text{OH} \rightarrow \text{Ag}(\text{NH}_3)_2\text{OH} + 2\text{H}_2\text{O} + \text{NH}_4\text{NO}_3$ . This silver solution worked excellently, using the balance of Gomori's technic. It was at this point that I wrote Gomori asking for an emendation of his directions for preparing the silver solution.

As finally developed our proposed technic follows this sequence.

1. After formalin fixation, run paraffin sections through xylene to 100% alcohol.

2. Immerse in 1% collodion in 100% alcohol and ether, equal parts, 5 to 10 minutes.

3. Drain 1 minute.

4. Place in 80% alcohol, 5 minutes (can wait indefinitely here).

5. Wash in tap water.

6. Apply 0.5% potassium permanganate, 2 minutes.

7. Wash in water.

8. Treat with 5% oxalic acid, 2 minutes.

9. Wash in water.

10. Apply official solution of iron chloride (Liquor Ferri Chloridi U.S.P. XI) 1 ml., distilled water 49 ml., 2 minutes. With Orth fixation omit this step.

11. Wash in tap water, 3 minutes.

12. Wash in distilled water, 2 changes.

13. Immerse in di-ammine-silver hydroxide solution 3 minutes. To prepare: Place 1 ml. 28% ammonia water in a small flask and add 7 or 8 ml. 10% silver nitrate rapidly, then continue to add 10% silver nitrate drop by drop shaking between each addition to clear the solution until a faint permanent turbidity remains after the last drop added. This takes a total of 9 to 10 ml. silver solution. Then dilute the resultant solution with an equal volume of distilled water. This solution can be used for at least two days, but is not used more than once.

Place slides on glass rods face up over a suitable slop jar and drop about 2 ml. of the silver solution on each slide. After 3 minutes

14. Drain and rinse quickly with distilled water.

15. Flood slides with 10% formalin for 2 minutes.

16. Wash in running water 3 minutes.

17. Tone in 0.2% acid gold chloride 2 minutes.

18. Rinse in water.

19. Fix in 5% sodium thiosulphate 2 minutes.

20. Wash in water.

21. Counterstain as desired; e. g., with acetic alum hematoxylin (0.5%) for 2 minutes, followed by washing 2 minutes in water, then with 0.5% eosin or Van Gieson's picro fuchsin for 1 minute, or with an acid fast stain, or with a simple basic anilin dye such as 0.1% aqueous thionin, safranin or toluidine blue.

22. Dehydrate with 95% alcohol after the Van Gieson stain, and then, after all methods, with 3 changes of acetone, which removes the collodion film, and clear with acetone and xylene, equal parts, followed by 2 or 3 changes of xylene.

In this technic the ferric iron mordant before the silver still causes alum hematoxylin to stain nuclei black, and it is sometimes preferable to omit the hematoxylin or possibly to substitute 3% hydrogen peroxide for the ferric salt. Usually a quite complete impregnation of reticulum, and little silvering of other structures are obtained.

On comparative trial it was found that, on formalin-fixed material, omission of the permanganate oxidation of step 6 resulted in failure of impregnation. Omission of the oxalic acid reduction gave rise to a number of failures of impregnation, though some sections showed excellent reticulum.

The ferric chloride (step 10) is necessary for regularity of impregnation of formalin material, though good impregnation may often be obtained without it. In ferric iron content, the ferric chloride corresponds approximately to Gomori's 2% iron alum solution, and was adopted as simpler to prepare. Wilder's 5-to-10-second bath in 1% uranyl nitrate in place of the ferric chloride also gives excellent results.

I must dissent from Wilder's and Gomori's views that this step constitutes a "sensitization" by metal impregnation. Gomori also reported good results with silver, gold, cadmium, lead, tin, and uranium salts. He specified ferric salts in the iron group, but did not say whether plumbic and stannic salts were required. Silver and gold salts are readily reducible, and hence can be regarded as oxidants as are ferric and hexavalent uranium salts. That these salts act as oxi-

dants is suggested by the fact that 3% hydrogen peroxide, 1% sodium iodate, 5% potassium bichromate in 1% acetic acid, and 1% iodine in 2% KI solution could each be substituted for the ferric chloride or uranyl nitrate with quite good to excellent results.

It must be noted that with material primarily fixed in Orth's fluid this step with ferric chloride or other oxidant should be omitted. With this fixation washing with distilled water after the oxalic acid and then silvering give good to excellent results, and introduction of any of the oxidants used successfully with formalin-fixed material tends to engender much precipitate and interfere with the impregnation of reticulum. Only the acetic acid potassium bichromate solution was well tolerated, and it afforded no improvement over direct passage from oxalic acid through water to silver.

The silver solution was tried with good results without adding water. At this concentration 1 minute sufficed. Dilution with an equal volume of water and prolongation of silvering to 2 or 3 minutes seemed to give slightly more consistent results. Higher dilutions gave irregular and inconsistent results in the times tried. Variation of the time in the 50% dilution (1.5, 3 and 6 minutes) made no important difference. However the best time was perhaps 3 minutes.

Various dilutions of strong formalin were tried in step 15. A 1% dilution gave fairly good results with 4 minutes reduction, 2% formalin was irregular in its action, 5% was quite good in general, but there were irregular unsilvered areas in some sections; 10% and 20% formalin for 2 to 4 minutes generally gave good results, while 1 minute was often inadequate.

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## THE USE OF CLARITE IN PREPARING WHOLE MICROSCOPIC MOUNTS

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The usual difficulties encountered in the use of glycerin jelly or balsam as the mounting medium in the preparation of whole microscopic mounts can, in a large measure, be overcome by the use of Clarite or Clarite X,<sup>1</sup> both cycloparaffin or naphthalene polymers which have found use as histologic mounting media.<sup>2</sup> Several descriptions of such resins for use in microscopy have found their way into the literature lately and their increased popularity as well as the results obtained should prove interesting.<sup>3</sup>

These polymers prove especially useful where microprojection demonstrations are desired or where prolonged microscopic study is necessary. This is due to their high melting point which can stand the heat of an arc lamp very much better than glycerin jelly, Canada balsam or gum damar.

The Clarite whole mount technic is similar to that involved were glycerin jelly used as the mounting medium.<sup>4</sup> For larger specimens some sort of cell is required and the hollow ground slide (cavity slide) similar to those used in the examination of living bacteria has shown distinct advantages. Its greater depth allows specimens as large as 1 mm. to be mounted, while because of the high refractive index of Clarite, no noticeable aberration results. In the case of arthropods, where difficulty is encountered in penetrating the chitin, fixatives of a great penetrating power are used. The sublimate fixatives work well, particularly the Petrunkevitch modification of Gilson's Fluid<sup>5</sup> which is efficient in regard to its time and end effect on the specimen. The following method has been most successful in the case of *Anoplurida* and *Acarinida*.

<sup>1</sup>Courtesy of the Neville Co., Pittsburgh, Pa.

<sup>2</sup>Groat, R. A. 1939. Two new mounting media superior to Canada balsam and gum Damar. *Anat. Rec.*, **74**, 1.

<sup>3</sup>Richards, O. W., et al. 1943. Plastic coverglass substitutes in microscopy. *Abs. in Anat. Rec.*, **87**, 4.

<sup>4</sup>Para, M. 1944. The application of a processed glue as a substitute for glass coverslips in histologic technique. *J. Lab. and Clin. Med.*, **29**, 1294-9.

<sup>5</sup>Lee, A. B. 1937. *The Microtomists Vade-mecum*, 10th Ed., edited by J. B. Gatenby. Blakiston's, Philadelphia. See pp. 223-4.

<sup>6</sup>Gilson Fluid (Petrunkevitch modification) Water, 300 ml.; glacial acetic acid, 90 ml.; absolute alcohol, 200 ml.; nitric acid, 10 ml.; sublimate to saturation.

To detach any debris which may adhere to the specimen, it is washed in running water over night. The specimen is then killed and fixed in hot Gilson's Fluid (Petrunkevitch modif.). This should continue for fifteen minutes after which the specimen is washed for one half hour in 70% alcohol. Dehydration is carried out in alcohols of 70%, 80% and 95%, the exposure being thirty minutes for each strength.

Following dehydration the specimen may be kept indefinitely in xylene. At this point staining can be accomplished but caution should be exercised in selection of the schedule since alcoholic stains must be used.

Permanent mounts are made by filling the cavity of the hollow ground slide with a 60% Clarite-xylene solution in excess. The specimen may be manipulated with xylene-dipped needles and thus properly centered. The cover slip is then applied and it will be found that the excess xylene will form a perfect seal around the entire cover slip. Following application of the cover slip, the slide should not be touched for twenty minutes. After this time, the slide may be used normally but care must be taken not to store the slide on its side since, while an air tight seal is formed between the cover slip and the slide, the Clarite in which the specimen is mounted will not be sufficiently hard for at least three weeks and the mounted specimen would become displaced were the slide stored on its side.

TABLE 1.\*—COMPARISON OF THE TYPICAL PROPERTIES OF CLARITE AND CLARITE X WITH GUM DAMAR AND CANADA BALSAM

	Clarite	Clarite X	Damar	Canada Balsam
Melting Point C°.	145-155	145-155	105	61
Refractive Index $n_{25}^{\circ}$	1.544	1.567	1.524	1.535
Color	water white	same	pale amber	dark amber
Effect of Age on Color	stable	stable (yellows slightly)	yellows slowly	yellows rapidly
Acid No.	none	none	32.2	101.3

\*Courtesy of the Neville Co., Pittsburgh, Pa. December, 1939.

## NOTES ON TECHNIC

### STERILE MILK FOR BACKGROUND IN CAPSULE STAINING

In an article entitled "Staining Bacteria and Yeasts with Acid Dyes" (Stain Technology, 16, 13, 1941) W. E. Maneval describes a staining solution which contains an acid dye in combination with phenol, ferric chloride, and glacial acetic acid. He suggests its use in the demonstration of capsules, with albumen as a background material.

Sterile litmus milk, however, proves better for the purpose than albumen. In using milk, one places a drop of the sterile fluid on a clean, grease-free slide, and adds to it a small amount of the culture to be tested. The drop is allowed to dry and is then covered with Maneval's Solution A for five minutes. The stain is then washed off, and the smear blotted and dried.

This procedure is simpler than the Huntoon Method, as given in page IV-20 of the Manual of Methods for the Pure Culture Study of Bacteria, and the materials for its use are generally on hand in even the smallest laboratory. The Maneval solution contains not only the dye, but also the fixatives for the casein film.

I have used this method in connection with the study of capsulated bacteria associated with paper mill slime, but have not had the opportunity of trying it on other types of organisms.

Recently an unidentified contaminant has been encountered, in the form of large round globules, the source of which was not determined. It proved, however, that they existed on the surface of the film, and could be wiped off with xylene without disturbing the stained preparation.

Maneval's Stain, when made with more than one dye in the same solution, contains possibilities as a differential poly-stain, as claimed by the author, but I have not been able to determine just how these mixtures work with bacteria. Acid fuchsin and fast green FCF are very good dyes to use, either singly or in combination.—GLEN C. WEBER, 41 S. Church St., Keyser, West Virginia.

### FURTHER OBSERVATIONS ON ETHYL ALCOHOL SUBSTITUTES

A recent paper by the authors (Stain Techn., 20, 115-17, 1945) reported satisfactory results with various staining solutions made up with isopropanol or methanol in place of ethanol as a solvent. At the time of writing, none of the solutions were more than four

or five months old. At present the solutions have been kept eight to eleven months and a report on their relative keeping qualities can be given.

In this respect there has been essentially no difference between the solvents. All of the carbol fuchsin solutions have decomposed, even those made up with ethyl alcohol. Gelatinization has been observed in the case of carbol crystal violet; but this, as explained elsewhere (Conn, *Stain Techn.*, **21**, 21-2, 1946), is caused by too great concentration of the dye. All the other solutions that have been kept are still good, regardless of the solvent, except that Delafield's hematoxylin made up with methyl alcohol, C.P., Baker, proves distinctly inferior in staining qualities. (The similar solution made up with "Bioid" anhydrous methyl alcohol, Will, is still perfectly satisfactory.) Accordingly, the conclusions concerning these solvents as expressed in the previous article do not need to be modified after the solutions have aged. There is still no explanation available as to why there is a slight difference in the quality of the solutions made up with the two different grades of methanol.—H. J. CONN and MARY A. DARROW, *Geneva, N. Y.*

## LABORATORY HINTS FROM THE LITERATURE

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A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS  
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

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### MICROSCOPE AND OTHER APPARATUS

ULLRICH, H. Über die Bedeutung anastigmatischer Beleuchtung für die Polarisationsmikroskopie schwach doppelbrechender Objekte. *Zts. Wis. Mik.*, 59, 8-11. 1943.

The author stresses the significance of anastigmatic illumination in polarization microscopy of feebly double refracting objects. Comparison is made in results obtained with the ordinary attachable Nicol prism polarizers and analyzers as used on biological microscopes with the newer filter-polarizers of the "polaroid" type. The latter appear to be very superior in their performance and give better results than some of the older, special mineralogical microscopes.

In addition to the inherent astigmatism produced by the Nicol prisms above mentioned, attention should be given to the source of illumination, i. e., the type of lamp used, whether direct or angular illumination, to eliminate the secondary polarization produced by the mirror. Monochromatic light obviates most of the difficulties but makes observation very tiresome and frequently results in erroneous interpretation.

To obtain best anastigmatic polarization it is recommended that the following rules be observed: 1, illumination should be in the direct axis of the microscope (an opal glass at the light source will improve results if a low voltage filament is used as the light source); 2, maintain a numerical aperture of the illumination slightly less than that of the objective, as far as it is compatible with the desired resolution; 3, avoid the use of oculars which converge considerably; 4, the analyzer should be in the body tube of the microscope. (Note: the disturbances produced in analyzers with Nicol prisms over the eyepiece are always noticeable.)—*J. M. Thuringer*.

WICHTERMAN, RALPH. A new glass device for staining cover-glass preparations. *Science*, 103, 23-4. 1946.

The author describes a glass device which makes possible the staining of ten cover-glass preparations in a single operation without the dangers to the preparations inherent in ordinary methods. The apparatus is fully described with an accompanying figure.—*T. M. McMillon*.

### PHOTOMICROGRAPHY

GRÄBNER, K. E. Graphische Darstellung der Filterfunktionen. Ein Hilfsmittel für die Mikrofotografie. *Zts. Wis. Mikr.*, 59, 12-21. 1943.

The author presents a graphic demonstration of filter function as an aid to photomicrography. Directions are given for the preparation of photographic filters, also a table of the more important filters and filter combinations with their relative exposure factors. The interpretation of the gray scale and the preparation of simplified "Logario-tables" (sensitivity curves) are likewise described.—*J. M. Thuringer*.

HAUSER, E. A., and LE BEAU, D. S. Light-field ultramicrophotography of lyogels. *J. Amer. Chem. Soc.*, 68, 153-4. 1946.

Since color photographs are superior to black and white film for recording fine microstructures, Kodachrome pictures were used as negatives in making enlargements on regular printing paper.—*R. T. Whittenberger*.

MURRAY, NELSON A. A combined 35 mm. and cut film photomicrographic apparatus. *J. Techn. Methods*, 24, 46-50. 1944.

The author describes an inexpensive photomicrographic apparatus which is much more satisfactory than the optical bench apparatus it replaces. Any

camera using a single film holder can be used. The 35 mm. adapter is arranged so that it can be removed from the machine between exposures. The microscope rests on a platform, below which is another platform for the lights. A telescoping back is set with screws so as to insure a sharp image and the adjustment need never be changed. A 10-inch projection distance fills a  $3\frac{1}{4}$  by  $4\frac{1}{4}$  inch frame and at the same time gives an image size corresponding to the magnification indicated by the combination of microscope lenses used.—*Challiss Randall*.

### MICROTECHNIC IN GENERAL

**KRÜGER, F.** Die Möglichkeit der direkten Übertragung von Objekten aus Alkohol in Paraffin. *Zool. Anz.*, 137, 226-30. 1942.

The author reports some preliminary attempts to embed tissue in paraffin directly from absolute alcohol. The intermediate steps include the maintenance of a saturated alcoholic atmosphere about the paraffin container together with the addition of absolute alcohol to the melted paraffin. This process of embedding is much slower than the customary methods and the results do not appear to justify the elaborate precautions that must be observed to secure even a moderate degree of success.—*A. B. Dawson*.

### DYES AND THEIR BIOLOGICAL USES

**BUGYI, BLASIUS.** Weitere Beiträge zur kapillaranalytischen Reinheitsprüfung der histologischen und bakteriologischen Farbstoffe. Zugleich Erwiderung auf die Bemerkungen von Kocsis zu meinem Aufsatz "Reinheitsprüfung der histologischen Farbstoffe". *Zts. Wis. Mikr.*, 59, 1-7. 1943.

The author urges the routine use of capillary analytical methods in the examination and periodical checking of stains and chemical reagents in histological and bacteriological laboratories. The methods are based largely upon the chromatographic technic originated by Platz-Neugebauer with modifications introduced by Danckwortt, Cocsis, Meyer and the author. The impression is given that the method is simple, that it may be entrusted to relatively inexperienced laboratory personnel and lastly that none other than the usual equipment on hand in such laboratories is needed. Eventually the author concedes that a Hanovia quartz U.V.L. generator with filters and a Zeiss "Pulfrich" Stufenphotometer would add greatly to the accuracy of the determinations.

The article is, as indicated in the title, principally polemic in nature giving no details of procedures.—*J. M. Thüringer*.

### ANIMAL MICROTECHNIC

**MANWEIL, REGINALD D.** The J.S.B. stain for blood parasites. *J. Lab. & Clin. Med.*, 30, 1078-82. 1945.

The J.S.B. stain (Jaswant Singh and L. M. Bhattacharji. *Indian Med. Gaz.*, 79, 102, 1944) is described in detail. The author considers it superior in most respects to the other commonly used processes for staining blood and blood protozoa. Preparations are much like those obtained by the Giemsa method, in definition, clarity and color values, but slightly less resistant to light. The technic is simple and rapid. The solutions are inexpensive, and stable for many months.—*John T. Myers*.

**NYKA, W.** A histological study of the lungs of mice infected with typhus rickettsiae. *J. Path. & Bact.*, 52, 317-24. 1945.

Rickettsiae in the lungs of mice may be demonstrated by the following procedure: Fix in 10% neutral formol. Stain 30 min.—1 hr. in 1:10,000 aqueous methyl violet. Differentiate in weak acetic acid (2 drops glacial acetic acid in 100 ml. water) until all cytoplasm is unstained. Counterstain with 1:10,000 aqueous metanil yellow for a few seconds. Dehydrate with acetone, clear in xylene, and mount in a neutral medium. Rickettsiae stain violet.—*S. H. Hutner*.

**PHILLIPS, PAUL H.** A practical method of coloring semen for identification purposes. *J. Dairy Sci.*, 28, 843-4.—1945.

A method is described for using a series of stains and dyes to differentiate the semen of various breeds used in the artificial insemination of dairy cattle.

Fresh semen was diluted 1:5 with the fresh egg yolk buffer. Five per cent solutions of methylene blue, Nile blue sulfate, thionin, Janus green, alizarin sulphonate, neutral red and Sudan III were used. Enough of each dye was added, drop by drop, to color the sample. It was impossible to dissolve the Sudan III in aqueous solution and a suspension of this dye was used. The diluted semen samples were stored in the refrigerator at approximately 5° C. Daily microscopic observations were then made on each treated sample until differential motility ratings were established.

Nile blue sulfate, neutral red and Sudan III caused no appreciable detrimental effect upon motility of the semen for several days. Sudan-III-treated samples were superior to yolk buffer alone in maintaining a high degree of vigor and motility. It appeared that the Sudan III through its affinity for free fat and its solution in the fat droplets actually improved the storage medium. This dye was inferior because its insolubility prevented the development of even an average tinge to the diluent. The other dyes tried were not satisfactory, although Janus green could be used for a limited time if necessary.

At the time these observations were made actual breeding tests could not be used as the final and more desirable evaluation.—*William G. Walter.*

VON SCHEVEN, RICHARD. Ein Beitrag zur Frage der Erythrozytenmembran. *Zts. Wis. Mikr.*, 59, 35-9. 1943.

For demonstrating the membrane of erythrocytes, the author proceeds as follows: Add 1 ml. freshly drawn venous blood to 50 ml. of a 0.3% solution of NaCl, agitate and allow to stand for 15 hr. Centrifugalize portions of this in regular centrifuge tube. Pour off supernatant fluid. Add to the sediment an equal amount of fixing and staining fluid prepared as follows: 1 drop of a mixture of equal parts of pyroligneous acid, methyl alcohol and formalin added to 8 drops of the stain (2 ml. Breslau's opal-blue-phloxine-rhodamin, 1 ml. liquified phenol, 17 ml. glycerin, 60 ml. distilled water) and allow to stand 2 hr. after thorough shaking. This fluid does not overstain. To examine, smear a small drop on slide; cover and examine with oil immersion lens. The wrinkled appearance of the erythrocytes is explained by the leached contents of the erythrocytes being partly replaced by the fixing and staining fluid.—*J. M. Thuringer.*

### PLANT MICROTECHNIC

MEITRES, MARC. Heterogeneite structurale de chondrioconte chez *Lupinus albus* L. *Compt. Rend. Acad. Sci. Paris.*, 218, 241-3. 1944.

Chondriosomes are not homogeneous structures but are actually composed of chains of small granules. To demonstrate this structure, root tips are killed by the IVA technic of Regaud (see Meitres, C. R. Soc. Biol., 137, 225, 1943), with an added wash in a fat solvent between the treatment with formol and bichromate. The detailed fixing technic is as follows: Root tips are placed in 20% neutral formal for 6 days; then transferred for 72 hours to a solution made by mixing 10 ml. water, 20 ml. 96% alcohol, and 100 ml. benzene,—this is shaken and the aqueous phase discarded; a wash of 30 minutes is followed by a post-chromatization according to the technic of Dietrich-Parat.—*T. E. Weier.*

### MICROÖRGANISMS

CLANCY, CARL F., and WOLFE, DON M. A rapid staining method for *Rickettsia orientalis*. *Science*, 102, 483. 1945.

The method for staining *Rickettsia orientalis* in smears from animal tissues and providing differentiation from the tissue background is as follows: Smears of infected yolk sac membranes or other tissues are prepared, air dried, and fixed by heat. The slide is flooded with xylene, drained, and after drying in a current of air, is immersed for 5 min. in a distilled water solution of methylene blue 1:5,000 and basic fuchsin 1:5,000. The preparation is then washed in tap water, dried and examined. The dilute stain is readily prepared from 1% stock solutions of each of the two dyes and should be prepared daily to obtain best results. Smears thus stained show *R. orientalis* as a blue organism on a pinkish purple background.—*T. M. McMillon.*

JASMIN, A. M. An improved staining method for demonstrating bacterial capsules, with particular reference to *Pasteurella*. *J. Bact.*, 50, 361-3. 1945.

To demonstrate capsules on cells of *Pasteurella mastitidis* and related forms, the following modification of Hiss's method was useful: Transfer the amount of culture picked up on a fine straight platinum wire from the surface of a culture to a loopful of physiological saline containing 0.5 to 1% phenol and 10% blood serum; spread into a thin film on a clean polished slide; fix the dried film with a rapid dip into methyl alcohol; drain and flame to burn off excess alcohol; stain 30 sec. to 1 min. in any common bacterial stain; wash with water. The capsules appear as clear areas against a lightly stained background while the bacteria are darkly stained.—*Virgene Kavanagh*.

ROGERS, T. H. The inhibition of sulfate-reducing bacteria by dyestuffs. Part II. Practical applications in cable storage tanks and gas holders. (Appendix by E. C. Barton-Wright on application in a flour mill.) *J. Soc. Chem. Ind.*, 64, 292-5. 1945.

A previous investigation showed that sulfate-reducing bacteria could be inhibited by dyestuffs of the flavine type. This paper describes the application of one of these dyes in preventing the corrosion of submarine cables and the fouling of gas holder waters and a wheat washing plant by bacteria producing  $H_2S$ . Dye No. 914, a derivative of 3, 6-diaminoacridine prepared by Imperial Chemical Industries, Ltd., was used usually at a concentration of 1 part in 250,000.—*R. T. Whittenberger*.

### HISTOCHEMISTRY

GULBRANSEN, R., PHELPS, R. T., and LANGER, A. A use of the electron microscope in chemical microscopy. *Ind. Eng. Chem., Anal. Ed.*, 17, 646-52. 1945.

Possibilities that the electron microscope may offer in chemical microscopy are discussed and a technic for adapting the microscope to this study is presented. The technic for the controlled growth of crystals on thin plastic films is based on the diffusion of ions and molecules through the film and subsequent precipitation on the film.—*R. T. Whittenberger*.

# STAIN TECHNOLOGY

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## PROGRESS IN THE STANDARDIZATION OF STAINS

TWENTY-FIVE YEARS OF COOPERATION  
WITH THE U. S. DEPARTMENT OF AGRICULTURE

As 1946 has witnessed the termination of formal coopération between the Stain Commission and the U. S. Department of Agriculture, this seems a good time to review the relationship which has existed between the two bodies for a full quarter century. This coopération started even before the Stain Commission was formally organized, and has continued ever since. Even now that it has officially come to an end, it is expected that cooperation with individual members of some of the Department Laboratories will continue.

### BEGINNINGS OF STAIN INVESTIGATIONS IN THE UNITED STATES

In 1920 the inadequacy of biological stains on the American market was beginning to be realized, and at least two independent attempts to do something about the subject were undertaken. The first of these was a letter written by R. T. Will of the Will Corporation of Rochester, New York to the Color Laboratory of the U. S. Bureau of Chemistry at Washington, asking whether they had "undertaken any work with the thought of assisting scientific investigators in pathological, biochemical and bacteriological fields in the preparation or the purification of dyes of American manufacture for scientific purposes"; also, whether they were "able to undertake, either the manufacture of new dyes or purification of those available". This letter was very courteously answered by Dr. C. O. Johns, then Chemist in Charge of the Color Investigation Laboratory, stating his interest, but also expressing his fear that nothing could then be accomplished along such lines, owing to a decreased appropriation and the resulting cut in personnel.

While this correspondence was going on, an entirely independent set of inquiries was being made on the part of the Society of American Bacteriologists as to whether there might not be some way of investigating and improving American stains. As a result, Dr. A. P. Hitchens, then Secretary-Treasurer of that Society, approached the writer, as chairman of the Committee on Bacteriological Technic, to see

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whether it might not be a matter within the scope of that committee. The writer agreed that it was, but mentioned practical difficulties in the way of organizing such an investigation.

A little later that year (October), by a lucky chance, these two lines of attack were brought together. The writer began having trouble with a certain dye (rose Bengal) of American manufacture, and the letter of inquiry on the subject, addressed to the Will Corporation, came to the attention of R. T. Will. The latter promptly expressed his interest in seeing some sort of standardization of American stains; and was informed that this very matter had been referred to the Committee on Technic. Mr. Will, therefore, decided that this Committee offered more immediate opportunities of accomplishing his objects than did the Department of Agriculture. A coöperative project to investigate certain American stains was organized by the Committee, with the coöperation of the Will Corporation to secure the necessary commercial samples to investigate.

Mr. Will, moreover, had not been discouraged by his earlier unsuccessful efforts to secure the coöperation of the Department of Agriculture and began pursuing other lines of attack in trying to get the investigation put on a broader basis. This time, his line of approach was through the National Research Council, and it resulted more successfully.

#### ORGANIZATION OF THE WORK UNDER THE NATIONAL RESEARCH COUNCIL

During 1921, considerable correspondence was carried on relative to organizing work of this sort under the National Research Council. It proved that Dr. McClung, the retiring chairman of the Division of Biology and Agriculture, was much interested and had been trying himself to develop a method of producing hematoxylin more satisfactory than the American product of that day. The project was therefore referred to his successor as chairman of that Division, Dr. L. R. Jones. The Research Council had better facilities for organizing cooperative work of this nature than had any body previously approached. Most important for assuring its success was the fact that active coöperation of the Chemical Foundation and of the Department of Agriculture was secured.

By this time, the Color Investigations Laboratory was in charge of Dr. J. A. Ambler, who was invited to be present at the first conference called on the subject. This conference was held on November 5, 1921. The result of the conference was the appointment of a Committee on Standardization of Biological Stains, which operated for about one year under the Research Council and succeeded in get-

ting the work organized. This was done in very close coöperation with the Color Laboratory under Dr. Ambler. His Laboratory made a large number of chemical and spectrophotometric analyses of stains, both satisfactory and unsatisfactory, and the Committee endeavored to find some correlation between such findings and their performance in actual use.

#### ORGANIZATION OF THE STAIN COMMISSION

If it had proved possible, as at first hoped, to find some correlation between performance and chemical or optical properties, standardization might have been accomplished by drawing up chemical and physical specifications. The early investigations soon proved, however, that such a course was out of the question, at least for the time being; accordingly, the only practical course seemed to be to issue some form of certification to those manufacturers whose products proved satisfactory. Such a plan seemed eminently desirable to all concerned; but it clearly was not one of the functions of the Research Council to act as a certifying body for commercial products. It was accordingly decided that some independent body should be set up to serve such a purpose. Hence, the Commission was formed as an independent organization affiliated with the Research Council; the full name given to it was then the Commission on Standardization of Biological Stains.

In order to give this organization more weight of authority than that derived from the individuals composing it, the plan was adopted of having its Executive Committee composed of representatives of various national scientific societies. Dr. Ambler became a member of this committee, when it was first organized, as a representative of the American Chemical Society. Serving in this capacity, he was also unofficially a representative of the U. S. Department of Agriculture, and took charge of the coöperative work carried on at the Color Investigations Laboratory. At first, this cooperation was wholly informal, but it was later covered by a formal Memorandum of Understanding, signed by representatives both of the Department and of the Commission. This agreement has been renewed every year up to 1945, and has thus continued through several changes in the internal organization of the Department; hence, although there has been a continuity in personnel representing the Department, the agreement has formally been drawn up with first one subdivision and then another of the Department. Whatever the name of the subdivision, however, the coöperation has always been given heartily on the part of the Department, even though all the other of the original color investigations came to an end long ago, and of recent

years the logic of continuing the stain investigations as part of the Department's program has been open to question.

#### EARLY PHASES OF THE COOPERATION

When the work was first organized, the Commission had no financial resources, and the funds appropriated to it by the Chemical Foundation were merely enough to cover a small amount of clerical work and travel expenses. Under such circumstances, the Department of Agriculture offered not only the facilities of the Color Laboratory, but the services of the assistants necessary to do the chemical work. In a short time, in fact, they secured a well-trained young dye chemist, W. C. Holmes, who gave the greater part of his time, as an employee of the Department, to the studies of biological stains; and later he was assisted in this work by one or two others, notably, J. T. Scanlan.

The early phases of the work consisted largely of developing methods for analysis of stains, and the routine assay of samples submitted by the companies; it was obvious that the former undertaking had to precede the latter. Few methods were known at the beginning of the work, so they had to be developed for one or two dyes at a time, and no stain could be put on the certification basis until the preliminary work on methods had been done. As a result, the Color Laboratory was doing the lion's share of the entire work on stains during the first stages of the coöperation.

It should also be mentioned that the first Edition of *Biological Stains* was published in 1925. Dr. Ambler gave much assistance in writing that book, and can be regarded as most outstanding among the collaborators in that undertaking.

In the late twenties changes began to occur. Dr. Ambler left the work, and the laboratory of which he had been in charge became primarily devoted to the utilization of farm wastes, the color investigations becoming a minor part of its activities. For several years, however, the coöperation of the laboratory with the Stain Commission remained as active as ever; when Dr. Ambler left, the biological stain investigations were put in charge of Mr. Holmes, who became the American Chemical Society's representative on the Commission. In this capacity he became one of the Commission's most active members, and gave very valuable assistance not only in the analytical work but also in the revision of the book, *Biological Stains*. The second edition of this book appeared in 1929, and it was thanks more to him than to anyone else that it became a very much more useful contribution to the field than had the first edition; he gave invaluable services in ironing out errors that had occurred in the first edition,

largely because it had been written by individuals having so little personal knowledge of dye chemistry.

In 1930 the Commission for the first time employed a chemist of its own; this was Miss A. R. Peterson—now Mrs. Bradshaw. She was first hired to investigate certain special problems in which the Chemical Foundation was sufficiently interested so that they were glad to finance the additional expense; but after completing those investigations, she stayed on as the Commission's analyst. She did all her work in the laboratory which had been carrying on the Department's color investigations. At first, she was under Mr. Holmes' direct supervision, and in association with Dr. Scanlan. Mr. Holmes' sudden and untimely death in 1932 and a protracted absence of Dr. Scanlan, due to poor health, resulted for a year or so in putting the entire burden of the chemical work on Mrs. Bradshaw's shoulders. Later, as a result of the experience thus gained, she was able to take over the entire chemical work, although Dr. Scanlan's advice in regard to puzzling points has been in constant requisition ever since the time he recovered his health. During this period the representative of the American Chemical Society on the Commission was first Dr. H. T. Herrick who, as Chief Chemist in Charge of the Color and Farm Waste Division, was Dr. Scanlan's immediate superior; while later Dr. Scanlan himself became the representative and has served as such until the present year.

When later the Department was further reorganized and Dr. Scanlan's time was needed wholly in connection with their problems on the utilization of farm products, Mrs. Bradshaw assumed even greater responsibility in connection with this work. From that time on, the cooperation of the Department consisted chiefly in furnishing the Commission laboratory space and equipment; but although its contributions to the work did not any longer include the time of members of its staff (except in an advisory capacity), the cooperation still remained extremely valuable.

#### MOST RECENT PHASES OF THE COOPERATION

In 1940, the entire staff of what had once been the Color Laboratory was moved to Philadelphia as part of the staff of the Eastern Regional Research Laboratory, and the question came up as to what should then be done with the biological stain investigations, which hardly fitted into the program of that Laboratory. After much discussion, however, it was decided that the work was important enough so that the cooperation should be continued at least for the time being. As a result, the biological stain work was furnished laboratory space in the new building, and has been located

there for four or five years—after an intervening period of about a year, pending the completion of the Eastern Regional Laboratory, when the work was being carried on at the Philadelphia College of Pharmacy and Science. The coöperation has still been active during this period; and the location at Philadelphia had the advantage to the Commission of offering the advisory services of Dr. Scanlan who was a member of the Regional Laboratory staff.

Now the coöperation has come to an end, but with the best of feeling on both sides. The resignation of Mrs. Bradshaw last year has forced certain changes; and it seemed well to take the occasion to initiate other changes. The result eventually is going to be more complete centering of all the Commission activities in one place. It is with regret that the long association with the Department has been brought to an end; but making the change seems at present the wisest course.

This occasion is taken to express publicly the appreciation of the Commission to the Department of Agriculture for a quarter century of active and invaluable coöperation.—H. J. CONN.

# PRECOOLING COMBINED WITH CHROM-OSMO-ACETIC FIXATION IN STUDIES OF SOMATIC CHROMOSOMES IN PLANTS

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**ABSTRACT.**—The treatment of living excised roots in vials partially filled with water at 0°C. for 1½ hours before fixation in cold Benda fluid was found to shorten chromosomes and to preserve details of chromosome morphology in a number of plant species.

The recent announcement in this journal, by Hill and Myers,<sup>2</sup> of a technic in which cold treatment is used to contract somatic chromosomes, prompts the description of a somewhat similar technic that has been in use for some time in the author's laboratory. Roots are taken from young greenhouse-grown plants shortly after the tips emerge from the ball of soil within the pots. Straighter and more rapidly growing roots may be obtained if the plants are thoroughly watered and the pots removed and replaced a day prior to fixation. The removal of the pot provides an air space between the pot and soil into which the young roots rapidly grow. The required number of roots is removed from a plant by snipping off the exposed tips ½ to ¾ inch in length with a fine forceps. These are placed in a Syracuse dish containing water, and any adhering soil removed by means of small camel's hair brushes. The clean roots are then transferred, along with an identification number, to an open 12×50 mm. shell vial partially filled with distilled or pure spring water. The vial is immediately cooled by placing it in an ice bath at 0°C. For this purpose a glass battery jar containing ice cubes and water and placed in an ordinary refrigerator has been found convenient. The vials with water and living roots are partly immersed and held in an upright position in the bath in a rectangular board provided with a number of depression holes.

After a given period of precooling—an hour and a half has been found optimum for most materials—the water is poured off the roots and replaced with Benda fluid, also cooled to 0°C. The vials are

<sup>1</sup>On leave from Department of Genetics, Carnegie Institution, Cold Spring Harbor, N. Y.

<sup>2</sup>Hill, Helen D., and Myers, W. M. A schedule including cold treatment to facilitate somatic chromosome counts in certain forage grasses. *Stain Techn.*, 20, 89-92. 1945.

then stoppered and allowed to remain in the bath for an additional 2-3 hours, after which time they are removed to room temperature where fixation continues overnight. The Benda formula used contains 1% chromic acid, 16 ml.; 2% osmic acid, 4 ml.; and glacial acetic acid, 2 drops.

Dehydration, staining, and the application of pressure to the cover glass after mounting in balsam, to cause flattening and separation of the chromosomes, are the same as in a previously published schedule<sup>3</sup>. The crooked proximal portions of roots may be trimmed off with the needles at the time of embedding, and the meristematic tips aligned in parallel groups of 5 or 6 for cross-sectioning as a unit. The author generally prefers to place the roots of a single plant on a slide and does not glue them to cards, but this could be done if desired.

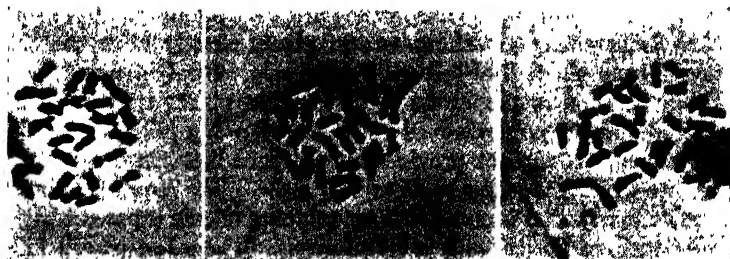


FIG. 1. Photomicrographs of somatic chromosomes from cold-treated roots of *Melandrium*,  $\times 1400$ .

The effect of low temperature in shortening somatic chromosomes was discovered accidentally during attempts to induce "nucleic acid starvation" by cooling whole plants, after the method of Darlington and La Cour<sup>4</sup>. Precooling the living roots in small vials makes possible the treatment of roots from large numbers of plants without extensive refrigeration equipment. It also permits a short treatment time to be used; since the roots rapidly attain the desired low temperature. The latter is an important consideration because the number of cells in division is not appreciably reduced by the treatment; chromosome shortening apparently is induced in cells already in the process of division. The fact that the roots are removed from the

<sup>3</sup>Warmke, H. E. A section-smear method for plant cytology. *Stain Techn.*, 16, 9-12. 1941.

<sup>4</sup>Darlington, C. D., and La Cour, L. Nucleic acid starvation of chromosomes in *Trillium*. *J. Genetics*, 40, 185-213. 1940.

plants before the cold treatment was found not to affect adversely the number of divisions or the fixation image.

Benda fluid is used because of its superior preservation of details of chromosome morphology, especially the centromere constrictions. The tendency of the chrom-osmo-acetic mixtures to give elongated and often overlapping somatic chromosomes, however, is completely overcome by the cold treatment. The combination of a fixing fluid that preserves details of chromosome structure, along with the shortening caused by cold, has proved tremendously useful in cytological studies of somatic chromosomes in this laboratory.

This technic has been used with excellent results on many different grasses, including species and varieties of *Panicum*, *Paspalum*, *Stenotaphrum*, *Penisetum*, and *Cymbopogon*, as well as on *Melandrium* and *Saccharum*. In *Melandrium* (Fig. 1) the centric constrictions are sufficiently well preserved so that it has been possible to make a study of spontaneous fragmentation of the Y chromosome and to correlate broken chromosomes with abnormalities in male development. These studies have shown that both arms of the Y may be involved, either singly or together, and that deficiencies may vary in length from small losses through a series of types to what appears to be the loss of a complete or nearly complete arm.



## A MODIFIED FEULGEN TECHNIC FOR SMALL AND DIFFUSE CHROMATIN ELEMENTS

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**ABSTRACT.**—A revised technic is proposed which differs essentially from the standard procedure only in the preparation of the staining solution and of the sulphurous acid bath, which are made by direct charging with  $\text{SO}_2$  gas instead of  $\text{HCl}$  and the sulphites ordinarily used. Tests on smear preparations of small amoebae, oöcyte prophases of a parasitic wasp, and yeasts revealed Feulgen-positive results not usually obtained by the conventional method.

### INTRODUCTION

In an attempt to study the behavior of chromatin in the mitotic sequence of small amoebae, the Feulgen technic given in McClung (1937) was employed; other methods (e.g. deTomasí's) were also investigated. However, except for the compact chromosome stages at metaphase, negative results usually were obtained. It was thought, therefore, that without altering the principles of the Feulgen reaction, some unessential and perhaps detrimental components might be removed from the usual reagents used and so a better reaction in the small, more diffuse chromatin elements difficult to stain would be promoted. Hence, a method was adopted which differs essentially from the standard technic only in the manner of preparing the staining solution and the rinsing bath. A decided improvement in delicacy of selectivity was immediately realized.

### METHOD

Hydrochloric acid apparently is necessary only to release sulphur dioxide from the sulphites ordinarily used in the formation of leuco basic fuchsin and in the sulphurous acid bath following staining. Both the acid and the sulphite were therefore eliminated, and direct charging of both the basic fuchsin and the bath water with sulphur dioxide gas was substituted. Normal hydrochloric acid was employed only in the necessary process of hydrolysis.

By gentle bubbling of sulphur dioxide gas from a small aperture in glass tubing into 100 ml. of 0.5% basic fuchsin solution, decolorization takes place in about an hour and the reagent is then ready for

use. Distilled water is similarly saturated for the sulphurous acid rinsing bath and may be stored for weeks in a tightly-corked flask. The sulphur dioxide gas can be produced easily by a simple flask-and-funnel generator using sodium bisulphite and dilute sulphuric acid.

The reagents thus made were then used according to the slightly modified standard procedure as follows:

1. Fixation, mostly 2 to 20 minutes. (Time is for smear preparations).
2. Washing, minimum time according to the fixative used (not over 20 minutes).
3. Distilled water, 2 minutes.
4. Normal HCl at room temperature, 2 minutes.
5. Normal HCl at 60°C., 8 to 10 minutes.
6. Normal HCl at room temperature, rinse.
7. Distilled water, rinse.
8. Sulphurous acid, 2 minutes.
9. Leuco basic fuchsin, 1½ to 2 hours.
10. Sulphurous acid bath, for sufficient time to remove the free unreacted leuco basic fuchsin (usually two or three 1-minute changes).
11. Tap water, 10 to 15 minutes.

Counterstaining with fast green may be in either aqueous or alcoholic solutions. Dehydration is accomplished either through the alcohols or from water through triethyl phosphate (Nelsen, 1945) directly into xylene. The latter is a shorter method and does not appreciably remove the aqueous counterstain.

### DISCUSSION

The foregoing method was tested on smear preparations of three types of organisms: (1) various small, endosome-containing amoebae; (2) germinal cells of female parasitic wasps, *Habrobracon*; and (3) the yeasts *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*. The fixatives used were: for amoebae, either Schaudinn's or weak Flemming's; for *Habrobracon*, formalin-acetic-alcohol; and for yeast, chrome-acetic-formalin. Fixation time was restricted to as low as two minutes and to not more than twenty minutes, with the exception of *Habrobracon* which was kept in the fixative for from one to about three hours. A minimum time of fixation and washing in water as well as a minimum preservation time in the alcohols was found most favorable. Also, a basic fuchsin which becomes decolor-

ized to a light straw or even a lighter hue proved to be best; see deTomasì (1936)<sup>1</sup>.

The different species of amoebae registered all the mitotic phases including very delicate and diffuse chromatin and so presented a complete picture of the mitotic series.

For *Habrobracon*, the staining method was used both by Dr. Anna R. Whiting of this laboratory and by the author. During the growth period, the oöcyte nucleus (prophase I) representing diffuse chromosomes did not stain by the conventional procedure (Speicher, 1936, and A. R. Whiting, personal communication), but did show up distinctly when the above described method was tried.

The yeasts, grown on Sabouraud's agar, clearly showed in the vegetative stages not only a densely-stained, Feulgen-positive "centriole" or "nucleolus", but also Feulgen-positive chromatin in the form of threads and granules in the "nuclear vacuole".

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<sup>1</sup>The lots of dye used most constantly in this work were a Grubler sample and one (Cert. No. CF-17) from Coleman and Bell. Four other batches, bearing certification numbers CF-5, CF-8, CF-27, and LF-7, were also employed. Three batches, NF-49, NF-52 and NF-53, which had been approved by the Stain Commission only for purposes other than the Feulgen stain, proved usable, but the color of the Feulgen-positive bodies was somewhat brownish, and the reaction was not sufficiently intense.



## AN OXIDATION AND ADSORPTION REACTION FOR DIFFERENTIATING THE ENDODERMIS AND THE COLLENCHYMA

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**ABSTRACT.**—A method is presented for a study of the endodermis and collenchyma in vascular plants. The method is useful in histologically isolating these tissues, particularly where the accepted anatomical features and the usual staining reactions are not definitive. The exclusive oxidation of the leuco (reduced) form of common redox indicators to the oxidized and colored form by the endodermis may be induced by treating fresh free-hand or freezing microtome sections with various alkaline salts at pH 8.5 to 9.0. Placing sections in sodium borate or sodium selenite induces an immediate oxidation of a tincture of gum guaiac in the endodermis and after several minutes a gradual and weak oxidation in the collenchyma. Addition of glycerol to sections which show oxidation in the endodermis induces a localized adsorption of the oxidized indicator in the collenchyma. The endodermis and the collenchyma may be differentiated best by oxidation and adsorption respectively by means of sodium selenite, guaiac and glycerol in the order given. The collenchyma in roots has not been defined to date, and the reactions described introduce a new method of study for this tissue. By treating sections with boric acid a gradient in the guaiac reaction appears in the cortex.

The unsaturated fat oxidation system of the endodermis has been described,<sup>1</sup> and the work presented here is an application of redox studies in isolating oxidases<sup>2</sup> to the problem of isolating the tissues noteworthy for their high content of unsaturated fat oxidase. Any oxidase indicator (guaiac, paraphenylenediamine, etc.) or any reduced (leuco) redox indicator will be oxidized initially and exclusively by the endodermis if sections are first treated with sodium selenite or sodium borate. Alkaline salts activate the oxidation of indicators by the endodermis, but other oxidation systems are depressed so that the localized oxidation reaction is a simple method of histologically isolating the endodermis.

<sup>1</sup>Van Fleet, D. S. 1942. The significance of oxidation in the endodermis. *Amer. J. Bot.*, 29, 747-55.

<sup>2</sup>Van Fleet, D. S. 1943. Unsaturated fat oxidase: distribution, function and histochemical identification in plant tissues. *J. Amer. Chem. Soc.*, 65, 740.

## PLATE 1

## Explanation of Figures

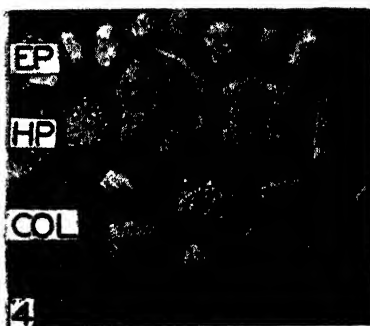
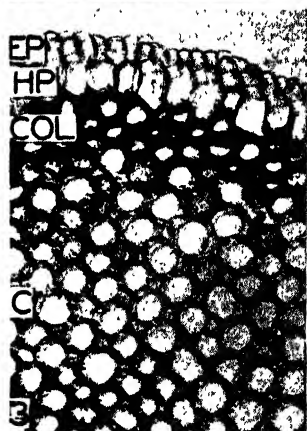
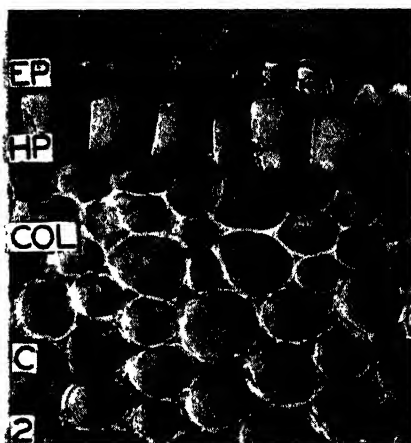
Photomicrographs of root sections ( $100\mu$  thick) of *Allium Cepa* var. *solanicum*.

FIG. 1. The dark shaded zone in the endodermis (EN) is the localized oxidation of gum guaiac following a one minute treatment with sodium borate. The xylem polar groups (XP) and alternate patches of phloem inside the stele do not react with guaiac until after 8-10 minutes, and the cortical cells (C) outside the endodermis do not react until 5-10 minutes after the initial reaction in the endodermis as shown in this photograph.  $\times 218$ .

FIG. 2. Epidermis (EP), hypodermis (HP), collenchyma (COL) and underlying cortical cells (C) unstained prior to reaction with gum guaiac. The characteristic histological features of the collenchyma (COL) with walls thickened in the corners may be seen as compared with the underlying typical cortical parenchyma cells (C).  $\times 295$ .

FIG. 3. The localized adsorption of gum guaiac in glycerol by the collenchyma (COL) is confined to this tissue and does not occur in the overlying dermal layers (EP and HP) or the cortical parenchyma (C).  $\times 190$ .

FIG. 4. An enlargement of the section shown in figure 3 to indicate the adsorption of oxidized guaiac in glycerol on collenchymal walls (COL).  $\times 300$ .



A 5% tincture of gum guaiac in 95% ethyl alcohol is boiled in Norit-A for 10 minutes to remove peroxides in the guaiaconic acid. A drop of gum guaiac tincture is applied to a fresh free-hand or freezing-microtome section and there is a generalized blueing (oxidation) of the guaiaconic acid by all of the cells in the section, but if the sections are treated for one minute with a 2% solution of sodium borate, sodium selenite, or sodium dibasic phosphate before applying the guaiac the reaction is confined to the endodermis for 5 to 10 minutes depending on the plant used. NaOH/HCl buffers at pH 8.5-9.0 will also induce a localized guaiac reaction confined to the endodermis (Fig. 1).

After standing in guaiac for 5 to 10 minutes there is a slow and gradual reaction in the collenchyma (Figs. 2, 3 and 4) that may be intensified by adding a drop of glycerol to the section and an additional drop or two of guaiac tincture. The addition of glycerol brings about the localized adsorption of oxidized guaiac (tetraguaiacolate) on the walls of collenchymal cells in root sections (Figs. 3 and 4). A differentiating stain for root collenchyma has not been described to date and the adsorption of oxidized guaiac by collenchymal cells introduces a new method of study for this tissue.

After sections have been treated with mild alkali and have been standing in guaiac for 10 minutes there is a gradual blueing of the cortical cells from the epidermis inward and from the endodermis outward. There is a gradient in this guaiac reaction in the cortex from the epidermis toward the center of the cortex. This gradient is best demonstrated by treating sections with boric acid (pH 5.6) followed by guaiac. After the initial reaction in the endodermis and collenchyma there is a cortical gradient reaction as follows: epidermis 1, 2, 3, 4, 5 — 5, 4, 3, 2, 1 endodermis. There are two waves of reaction, one from the epidermis inward, the other from the endodermis outward, and both meet in the center of the cortex, and they are mirror images.

In addition to the use of gum guaiac more careful studies may be made with common redox indicators applied in the colorless (reduced) form. Methylene blue, indigo carmine, 2-6 dichlorophenol-indophenol or brilliant cresyl blue may be reduced by means of sodium thiosulfate or sodium metabisulfite or by hydrogen gas bubbled through platinized asbestos. These indicators are more sensitive to free oxygen, and they must be applied under nitrogen in a hanging drop gas chamber or in a modified van Tieghem cell.

## A RAPID FREEHAND SECTIONING METHOD FOR LEAVES<sup>1</sup>

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**ABSTRACT.**—For the rapid sectioning of such material as fungus leaf spots a method has been evolved whereby a piece of leaf is soaked in lactophenol and sections are sliced off it, on the slide, under the dissecting microscope, by means of a diagonal scalpel ground with a slightly curved blade. Poor sections can be recognized and removed as soon as they are cut; and it is commonly possible at the same stage to distinguish which sections contain fruiting elements of a fungus.

During studies of the leaf smuts belonging to the genus *Entyloma*, and of other leaf parasites that require sectioning for adequate study, the writer has found that the preparation of a mount by sectioning in pith frequently takes longer than the examination of the mount and seriously limits the number of specimens that can be examined in a day. In addition, the method keeps the operator in some doubt as to the quality of the sections until the procedure has been completed, and the thinnest sections tend to be damaged by the requisite handling.

The method about to be described has been used by the writer for the examination of several hundred specimens during the last two years. It allows the complete preparation of a mount with a number of oriented sections, from selection of a lesion to application of the cover glass, in fifteen minutes or less. It also allows generous mounts to be made from a minimum of material, often an important consideration in the study of scanty and valuable specimens.

Presumably many workers have at one time or another tried to speed up section-cutting by some means, and some have probably used the dissecting microscope for the purpose. No doubt certain phases of the writer's method have been developed independently by others; but no description has been found in print of a similar process; and inquiry at several institutions has led to the belief that various features are sufficiently novel, and the advantages sufficiently great, to make publication advisable.

<sup>1</sup>Contribution No. 848 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.

<sup>2</sup>Junior Plant Pathologist.

A promising lesion is selected under the dissecting microscope, the area is soaked successively with 95% alcohol and water if dried material is being examined, and a piece is cut out. The size and shape of the piece will depend to some extent on individual preference and on the material examined. The writer generally uses a piece about  $5 \times 2-3$  mm., which may include the whole of a small lesion or an area cut radially from a large one to include a little healthy tissue at one end. If fruit bodies such as pycnidia can be seen upon the leaf, a strip is cut to include as many as possible. The piece is trans-

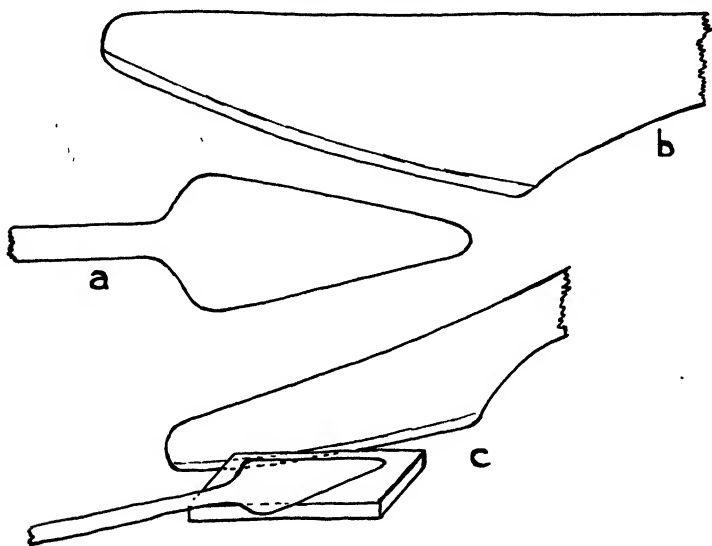


FIG. 1. a. Spear-point needle used to hold piece of leaf on slide.  
 b. Diagonal scalpel ground with slightly curved blade.  
 c. Positions of spear-point and diagonal scalpel during cutting of sections.

ferred to a drop of lactophenol at the end of a slide. The glass stage of the dissecting microscope is wiped clean, a drop of water or alcohol is placed in the center of it and the slide is pressed down upon it; this device holds the slide more firmly than do slide clips and leaves the whole surface clear for manipulation. The piece of leaf is drawn from the lactophenol drop toward the center of the slide, where it is briefly moved about to leave it in the middle of a thin film of the fluid.

The piece is now held down with a spear-pointed needle shaped somewhat as in Fig. 1a; this needle need not be sharp, but it should

be smooth and the edges of the blade should be approximately straight in order that one of them may be laid close to that side of the piece from which sections are to be cut. A diagonal scalpel is ground to a slightly curved edge as in Fig. 1b; the edge should be kept keen, but it is neither necessary nor desirable to attempt to bring it to the fine, smooth edge sought in a sectioning razor; a bevel of about  $20^\circ$ , finished and periodically renewed on fine abrasive paper, has been found satisfactory. The diagonal scalpel is used, as shown in Fig. 1c, to chop sections from the side of the piece that projects beyond the edge of the spear-point. The point of the blade is placed in contact with the slide beyond the end of the piece and the handle is lowered until a section has been cut off. The curved edge, used



FIG. 2. Freehand section of *Papaver orientale* showing spores of *Entyloma fuscum*, prepared by method described. Dried specimen. Photographed with 16 mm. objective.  $\times 130$ .

thus, cuts with a shearing action that improves the results; it also allows control of the scalpel during the cut, correction being easily made if the section is seen to be getting thicker or thinner. As each section is cut it is pushed away from the operator toward the far edge of the slide. The whole operation is carried out under the dissecting microscope. If a fairly high magnification is used (60 diameters has been found satisfactory) it is often possible to tell, as each section is made, whether it is useful; thus barren, thick or otherwise useless sections can be removed as made, and only usable sections are left on the slide. When there is some doubt as to whether the fungus is in good fruit, it is often advisable to cut a few sections, remove a thicker slice, cut more sections, and so on; thus the whole piece is sampled.

A point of some importance is the amount of lactophenol left on the slide; if too much is left both piece and sections tend to float out of position, whereas if the slide is practically dry the sections will be damaged as they are pushed across it. Surplus fluid is readily removed with the diagonal scalpel. The writer always keeps a sheet of hard lintless filter paper close to his right hand, for removing surplus lactophenol and pieces of leaf from the scalpel blade, and for cleaning scalpel and spear-point after completing a mount. Lactophenol can be added, if necessary, from the drop at the end of the slide.

Once sufficient sections have been cut and oriented, more lactophenol is added, a cover glass is applied, and the mount is ready for examination.

Figure 2 shows a section, prepared by this method, of the leaf of the oriental poppy, *Papaver orientale*, parasitized by the poppy leaf smut, *Entyloma fuscum*. The teliospores of the smut can be seen scattered in the intercellular spaces. The section was cut from a dried, unfixed, herbarium specimen.

In a wide variety of plants, attacked by various fungi and bacteria, little difficulty has been experienced in obtaining sections thin enough for critical examination with the oil immersion objective. The method is particularly useful for those firm, and often hypertrophied, lesions that are difficult to cut in pith. No claim is made that sections will be as thin as the best of those obtained from favorable material by the skilful use of a razor and pith. On the other hand uniformly fair sections are obtainable with very little practice, which is seldom true of the pith method. The time saved in preparing the mount, to say nothing of the time saved in sharpening a small scalpel as compared with that required for honing a razor, makes the method valuable in the type of work for which it was devised.

It is possible that slight modification would make this method useful for other purposes. Lactophenol has been used by the writer because it is a satisfactory mounting fluid for fungi and possesses the desirable characteristics of low surface tension, moderate viscosity and low rate of evaporation. Possibly mixtures of glycerol and water might prove adequate for the examination of living material; but the method will probably prove most useful with dried material, the firmness of which is preferable to the softness of many living leaves.

## SOME EVIDENCE FOR THE SPECIFICITY OF THE FEULGEN REACTION

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**ABSTRACT.**—Carr has attacked the specificity of the Feulgen reaction on three grounds: that the chromosomes are adsorbents capable of regenerating the color of the Schiff reagent; that selectivity for the nucleus depends on destruction of cytoplasm by acid hydrolysis preceding staining; and that the reaction is not blocked by SO<sub>2</sub> water, as he says it should be if staining occurs by a chemical reaction. The first point was tested by staining chromosomes treated with nuclease. They were Feulgen negative, but their protein basis remained intact. The second point was tested by hydrolyzing fixed tissues, washing off solutes, drying, and comparing weight loss with controls. As differences were negligible, the fixed cytoplasm must not have been made soluble by hydrolysis. Carr's third point was not tested experimentally. It is concluded that these objections to specificity of the Feulgen reaction are not valid.

Since the use of the Schiff reagent as a test for thymonucleic acid was first introduced in 1924 by Feulgen and Rossenbeck<sup>1</sup>, an extensive literature has appeared concerning its use. As Stowell<sup>2</sup> has recently reviewed this literature, it will not be necessary to review it here; however, most writers have held that, for practical purposes, the Feulgen reaction is specific, with only a few writers dissenting.

In a recent issue of *Nature*, Carr<sup>3</sup> has maintained that the Feulgen reaction is quite non-specific, and depends upon simple adsorption rather than upon the chemical reaction to which it is usually attributed. His argument rests upon three points. First, the Feulgen color is produced if alumina or other adsorbent is added to the Schiff reagent. As chromosomes ordinarily concentrate stains out of very dilute solutions, they may be regarded as good adsorbents. Hence it is likely that the Feulgen reaction is simply an adsorption. Second, biochemists ordinarily isolate nuclei from cytoplasm by hydrolysis with dilute acids. It therefore seems probable that the selectivity

<sup>1</sup>Feulgen, R., and Rossenbeck, H. 1924. Mikroskopisch-chemischer Nachweis einer Nucleinsäure usw. *Zts. physiol. Chem.*, **135**, 203.

<sup>2</sup>Stowell, R. E. 1945. Feulgen reaction for thymonucleic acid. *Stain Techn.*, **20**, 45.

<sup>3</sup>Carr, J. G. 1945. Mechanism of the Feulgen reaction. *Nature*, **156**, 143.

of the Feulgen reagent for the nucleus depends upon the destruction of much of the cytoplasm by the acid hydrolysis which precedes treatment with the Schiff reagent. Third, Carr says that, if a chemical reaction between the decolorized fuchsin and an aldehyde were responsible for the reaction, then it ought to be possible to block the reaction by pretreatment of the tissue with  $\text{SO}_2$  water. He tried this, using a great excess of  $\text{SO}_2$ , but the Feulgen reaction was given as usual. Each of these points will be separately discussed.

With regard to Carr's first point, it cannot be denied that the chromosomes are efficient adsorbents, but Carr himself admits that this does not exclude the possibility of a chemical reaction as the basis of staining. The question is, which of two possibilities actually does occur? Experiments of Mazia and Jaeger<sup>4</sup> on salivary gland chromosomes of *Drosophila* show that, if the tissue is first digested with nuclease to remove the thymonucleic acid, the chromosomes become Feulgen negative. Control slides, treated with boiled nuclease, give a typical Feulgen reaction. Staining the digested chromosomes with ninhydrin reagent shows that the protein skeleton of the chromosomes remains intact. Kodani<sup>5</sup> has extended this work to the lampbrush filaments which he has been able to demonstrate within the bands of the salivary gland chromosomes. Each "hair" has a nucleic acid component which can be removed by nuclease, and a protein skeleton which remains intact when the chromosomes are digested with nuclease. The digested hair is Feulgen negative, while the hairs of control slides remain positive. I have verified this work for the lampbrush chromosomes of amphibian eggs. Here again, nuclease action renders the chromosome Feulgen negative without disrupting the protein basis of the chromosomes. It would seem then, regardless of the mechanism of Feulgen staining, that a positive reaction does not occur in the absence of thymonucleic acid.

Carr's second point was also tested experimentally. Rat liver was fixed in Bouin's fluid, washed in 70% alcohol and hydrated, then ground up, and centrifuged to remove excess water. The mash was then divided into three samples, each of which was weighed. Sample A was then dried to constant weight. Sample B was hydrolyzed in 1 N HCl at 60°C. for 20 minutes, while sample C was treated with distilled water at the same temperature and for the same length of

<sup>4</sup>van Herwerden, M. A. 1913. Über die Nucleasewirkung auf tierische Zellen. Arch. Zellforsch., 10, 431.

<sup>5</sup>Mazia, D., and Jaeger, L. 1939. Nuclease action, protease action, and histochemical tests on salivary chromosomes of *Drosophila*. Proc. Nat. Acad. Sci., 25, 456.

time. Samples B and C were washed repeatedly to remove any soluble constituents, then dried to constant weight. Results are tabulated below:

Sample	Wet weight	Dry weight	Percent loss
A	3.914 g.	0.918 g.	76.5
B	3.742	0.849	77.3
C	5.622	1.053	81.3

As the differences in percentage loss are insignificant, it must be concluded that the efficiency of acid hydrolysis in destroying fresh cytoplasm does not extend to fixed tissue, and so selectivity of Feulgen staining cannot depend upon destruction of the cytoplasm. It may be objected that Bouin fixation should not be used in an experiment to test the effect of acid. The choice was made because Bouin's is commonly used before the Feulgen reaction. Also, it must be pointed out that if any cytolytic effect of Bouin's is sufficient to limit Feulgen staining to the nucleus, then practically all nuclear staining must be accounted for on this basis. It is doubtful whether anyone will care to accept this.

In making his third point, Carr starts from the usual assumption that hydrolysis of thymonucleic acid unmasks an aldehyde group. Treatment of such hydrolyzed thymonucleic acid with  $\text{SO}_2$  water should then cause the formation of an aldehyde-bisulfite addition product. However, these compounds are notably unstable<sup>6</sup>, particularly in the presence of dilute acid or alkali, so it is to be expected that the aldehyde-bisulfite addition product would be in equilibrium with an aldehyde-Schiff-reagent addition product. Because of the intensity of the stain, one would hardly expect to detect dilution of the stain by the usual visual methods. It ought to be possible, however, to find some reagent which would block the aldehyde group irreversibly, and thus give a decisive answer to this problem.

In conclusion, while our knowledge of the Feulgen reaction and its specificity leaves much to be desired, as yet no serious experimental evidence against its practical usefulness in the identification of thymonucleic acid seems to have been brought forth.

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<sup>6</sup>See any textbook of organic chemistry.



## CURTIS' SUBSTITUTE FOR VAN GIESON STAIN

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**ABSTRACT.**—A triple staining method is described in which nuclear staining is by Weigert's hematoxylin. The cytoplasmic and collagen staining is effected by the Curtis substitute for Van Gieson, in which ponceau S is substituted for acid fuchsin. Nuclear staining is sharper than with Delafield's hematoxylin. The red of the collagen fibers is probably not subject to fading. Unlike Van Gieson, this method gives staining of reticular as well as collagen fibers. The advantages of the method are its simplicity and reliability. The use of this method is made possible by a new source of reliable samples of the ponceau S called for in this method.

In 1889 Van Gieson described a method for staining cytoplasm and collagen differentially by the use of a mixture of picric acid and acid fuchsin. Most references given to this paper are indirect ones or references to abstracts of it.

Weigert (1904) introduced a new and excellent hematoxylin staining method specially designed to resist the acid differentiation occurring during counterstaining with Van Gieson. Curtis (1905) found that ponceau S could be substituted for the acid fuchsin of Van Gieson. Slides stained by his method are less liable to fade. But in Curtis' method, Delafield's hematoxylin was advised.

In this laboratory we have for some years (Carleton and Leach, 1938) used the Curtis' substitute after Weigert's hematoxylin and have obtained satisfactory results; but recently it has become impossible to get reliable samples of ponceau S. Owing to a confusion in nomenclature, completely different dyes were probably supplied.

At my request, Mr. Nantz of the National Aniline Division (Allied Chem. and Dye Corp.) kindly undertook to prepare a sample. I have tested this and find it as good or better than the samples that we used to employ with satisfaction. It is too early to say whether fading will occur.

The following is the method in use in this laboratory:—

1. Fix by any desired fixative.
2. Take sections down to water using iodine and hypo (sodium thiosulfate) if necessary.
3. Stain with Weigert's hematoxylin — 5 to 10 minutes.

Solution A. 1% hematoxylin in absolute alcohol.

Solution B.	30% ferric chloride in distilled water.	4 ml.
	Conc. hydrochloric acid.	1 ml.
	Distilled water	100 ml.

Mix 1 part of A with 1 part of B just before use.

Add 2 parts of distilled water.

4. Without acid differentiation, wash in running tap water 5 minutes.
5. Stain 2 to 4 minutes in Curtis' substitute:—
 

2% Ponceau S (National Aniline) C. I. 282	5 ml.
Saturated aqueous solution of picric acid	95 ml.
2% Acetic acid.	2 ml.

6. Rinse in 96% alcohol.

7. Dehydrate, clear and mount.

Results:—Chromatin — black.

Cytoplasm — yellow.

Collagen and reticular fibers — red.

The results are similar to those given by Van Gieson but both the red and yellow colors are purer and overstaining with the red is less likely to occur. This may be due to the molecular weight of ponceau S exceeding that of acid fuchsin (von Moellendorf, 1922). This would also be the reason why reticular fibers tend to stain by this method (Jaloway, 1937); this is an advantage unless differentiation of collagen and reticular fibers is desired.

The nuclear staining is good and suitable for photomicrography, although not as sharp as a Heidenhain preparation; it is better than after Delafield's which gives such disappointing results with Van Gieson.

This method has some advantages over the numerous other trichrome methods now in use.

- a. Simplicity and reliability. First year medical students have been known to get good results.
- b. It works well after all fixatives.
- c. It probably does not fade.
- d. It shows up masses of fibrous tissue clearly for naked eye and low power observation.
- e. The nuclear detail is good and in no way obscured by cytoplasmic staining. It is possible to get sharp photomicrographs of both nuclei and collagen fibers in one preparation.

The disadvantage is that cytoplasmic details are not as clearly revealed as after such stains as Masson or Mallory.

My thanks are due to Dr. H. M. Carleton and Mr. T. A. Marsland for their part in the evolution and testing of this method.

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## THE USE OF ENZYMES IN THE PREPARATION OF ROOT-TIP SMEARS

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**ABSTRACT.**—A simple root-tip smear method for the study of somatic chromosomes of *Allium* is described, in which an aqueous solution of colchicine and Pectinol, (an enzyme preparation) is used. The root tips are placed in a 0.2% aqueous colchicine solution for 30 minutes, fixed in propionic-alcohol solution, run through a graded series of alcohols, washed thoroughly, treated with a 1% aqueous solution of Pectinol for 1 to 1½ hours, and allowed to stand in water 3 to 5 days to soften. A thin transverse section through the meristematic portion of the root tip is then smeared on a slide in a drop of propiono-carmin stain in the usual way and sealed.

The use of enzymes in making smear preparations for the study of chromosomes has attracted considerable interest recently. Emsweller and Stuart (1944) described a technic by which enzymes derived from an extract of fungus mycelium and the commercial enzyme preparation Clarase were used in spreading pollen mother cell chromosomes of *Lilium* spp. Frolova (1944) used enzymes in studying the structure of salivary gland chromosomes. Fabergé (1945) found that a mixture of cytase and other enzymes obtained from the stomach fluid of the Roman or edible snail made an excellent macerating medium for smear preparations of root tips and other similar plant tissues. In the present study the commercial enzyme complex, Pectinol, has been used in an attempt to apply to root tips of *Allium* spp. a method similar to that of Emsweller and Stuart (1944) for pollen mother cells.

Because the root-tip chromosomes of *Allium* are long at metaphase, it is desirable in making counts to contract them by the use of colchicine, as described by O'Mara (1939), Burrell (1939), and Emsweller and Stewart (1944). Colchicine suppresses the formation of the achromatic figure so that more figures are obtained with metaphase chromosomes. This treatment not only shortens the chromosomes but makes the constriction region very conspicuous,

as shown in the accompanying illustration (Fig. 1). After fixation for one hour or longer in propionic acid and absolute alcohol, the root tips are carried through the alcohol series and the enzyme according to the schedule that follows. By this method the middle lamella of the cell wall is softened so that the cells separate when smeared on a slide in a drop of propiono-carminine stain. The condition of the cytoplasm seems to be changed to such an extent by the enzyme treatment that by applying very light pressure to the cover slip the cells are flattened and the chromosomes are spread out and separated from one another. Both the ease with which the root tips may be smeared and the sharpness of the stain after treatment with Pectinol are

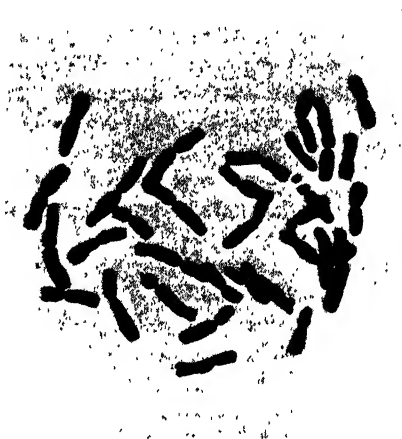


FIG. 1.—Division figure from a root tip of the amphidiploid species hybrid *Allium cepa* L.  $\times$  *A. fistulosum* L., showing 92 chromosomes after treatment with Pectinol.  $\times$  810.

considered by the authors to be superior to the results obtained by the use of the HCl method described by Warmke (1935).

Four different grades of Pectinol were supplied by the Rohm and Haas Co., Philadelphia, Pa. These are known as Pectinol A, W, M, and 100-D. The first three are for commercial applications while the 100-D is manufactured for experimental purposes only and is a standardized product. These Pectinols are derived from *Aspergillus*, and while their primary action is on pectins they also contain minute quantities of diastases and proteases. A 1% suspension of Pectinol 100-D, in distilled water, was used in this work. This suspension had a reaction of pH 6, and was satisfactory, even though the optimum pH is between 3 and 4.5. Pectinol 100-D is active at room tempera-

ture but its activity increases with rising temperature to approximately 49° C., above which point the activity decreases. In the procedure outlined Pectinols A, W, and M were as effective as the 100-D sample.

The detailed schedule is as follows:

1. Place excised root tips, about 1 cm. in length, in a 0.2% aqueous solution of colchicine for 30 minutes.
2. Wash in water for 15 minutes.
3. Fix in a solution of one part propionic acid to three parts absolute alcohol for 1 hour or longer.
4. Change to 95% alcohol for 15 minutes.
5. Change to 80% alcohol for 15 minutes. If desired the root tips may be stored indefinitely in the 80% alcohol.
6. For immediate study, run the material through a graded series of alcohols: 70, 50, and 25%, respectively, allowing 15 minutes in each. If a shorter schedule is desired, some of these alcohols could probably be omitted.
7. Wash thoroughly in two to three changes of distilled water during 30 minutes to 2 hours. Unless all traces of alcohol are removed, the Pectinol will be inactivated.
8. Place in a 1% aqueous solution of Pectinol for 1 to 1½ hours.
9. Wash in water and allow to stand at room temperature for 3 to 5 days, or until the root tips seem very soft when handled with forceps.
10. Place a thin freehand section of the treated root tip on a slide in a drop of propiono-carmin stain. Smear with a spear-pointed needle or other suitable instrument, cover, warm slightly over a flame, and press gently on the cover slip. If properly treated the root-tip cells should separate and float out in the stain drop as readily as pollen mother cells from an anther.

In cases where the larger root tips were not sufficiently softened to smear readily after the above treatment, they were placed again in a very dilute aqueous Pectinol solution (0.5%) for 1 to 4 hours. After rinsing in distilled water, they were in excellent condition for smearing.

11. For a temporary mount, seal with gum mastic and paraffin. If kept in a refrigerator the preparations are suitable for study for a week or longer.
12. To make the temporary mounts permanent, carefully scrape away the wax, float off the cover slip in equal parts of absolute alcohol and propionic acid, and mount directly in Euparal or Diaphane. This method of making slides permanent was recom-

mended by Burrell (1939) because it involves the least manipulation.

Pectinol was also tested in making smear preparations of pollen mother cells of triploid and tetraploid *Allium* spp. Anthers were fixed in the absolute alcohol and propionic acid solution and run through the alcohol series as described for root tips. After washing thoroughly in distilled water they were treated in 1% Pectinol for 30 minutes and smeared in a drop of propiono-carmin stain in the usual way and sealed. The results were as satisfactory as those obtained by the use of the Clarase method described by Emsweller and Stuart (1944).

The authors have worked almost entirely with a 1% solution of Pectinol but a recent trial indicates that a very dilute solution (0.1 to 0.05%) over a longer period may be equally effective.

The schedule given in this paper has been tested with *Allium* only, but presumably it may be adapted for use with the root tips and similar tissues of other plants.

The authors wish to express their appreciation to Dr. Marion W. Parker for suggesting the use of Pectinol in these cytological studies and for preparing and testing the enzyme extracts used.

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## LABORATORY HINTS FROM THE LITERATURE

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A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS  
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

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### MICROSCOPE AND OTHER APPARATUS

HAUSER, E. A., and LE-BEAU, D. S. Microscopic studies of lyogels. Preparation of samples for ultra-illumination by incident light. *Ind. Eng. Chem.*, **38**, 335-8. 1946.

The Ultropak ultramicroscope is used because it permits circular illumination of the preparation by incident light which does not pass through the lenses of the objective. Samples of rubber are dissolved and the solution is spread on a non-miscible liquid. A fine-mesh copper gauze is coated by being drawn upward through the rubber film. Preparations of capillary active substances like soaps are made by dipping the gauze vertically into the solutions. The gauze is permitted to dry and is placed on a microscope slide. Photomicrographs made by using color films are projected to give high magnification, and a tridimensional effect is apparent. Ultraviolet radiation enables the study of fluorescent materials.—*R. T. Whittenberger*.

VOLD, MARJORIE J., and DOSCHER, T. M. Hot stage for microscopic observations between room temperature and 350° C. *Ind. Eng. Chem., Anal. Ed.*, **18**, 154-5. 1946.

Commercially available heating stages generally are not serviceable much above 50° C. The hot stage described is constructed of porous fire brick and is suitable for use with 16 mm. and 8 mm. objectives. Temperature is measured with a thermocouple and temperature rise is controlled by means of a variac and a 12-ohm resistance. Temperature difference between center of hot stage and the outermost area varies only  $\pm 0.8^\circ$  C. at 250° C. A modification of the hot stage in which an air-driven fan is incorporated gives a temperature distribution that varies only  $\pm 0.3^\circ$  C. at 250° C.—*R. T. Whittenberger*.

### PHOTOMICROGRAPHY

LEONARD, DONALD W. Photomicrography: a short method using negative projection prints. *J. Lab. & Clin. Med.*, **31**, 373-8. 1946.

Negative projection photomicrographs can be produced with very simple equipment available in most laboratories. The microscope, the slide, and the light source should be arranged so that the image is projected onto the photographic paper, preferably contrast paper with a glossy surface. The print obtained will show a tissue section more nearly as it actually exists as it comes from the knife, than will the customary positive photomicrograph, i. e. a thin slice of blood-free white material outlined against a black background of space, its delicate pattern of cells and nuclei sharply visible.—*John T. Myers*.

### MICROTECHNIC IN GENERAL

BOURDON, P. L'utilisation des résines artificielles (polystyrène), comme milieu de montage, avec ou sans lamelles. *Bull. d'Histol. Appl.*, **19**, 175-8. 1942.

A previous article described the use of a cellulose varnish for mounting preparations which served both as a mounting medium and as a cover slip; but a commercial product was then used which was of indefinite composition and difficult to obtain. A commercial solution of polystyrene which had a very high molecular weight gave excellent results as a varnish, but was unsatisfactory for use with a cover slip because it lost 4/5 of its mass on drying. However, by partial polymerization of styrene, a product was obtained which was satis-

factory for both purposes. It is prepared as follows: boil gently 100 ml. styrolene until the liquid becomes of a consistency similar to glycerine; extinguish the flame and add 5 ml. of tricresyl phosphate; stopper the flask and chill rapidly. The liquid obtained is colorless, has a refractive index of approximately 1.53 and a viscosity comparable to that of Canada balsam. When used without a cover slip, this medium gives excellent preservation of color.—*Jean E. Conn.*

**BOURDON, P.** Sur l'emploi de la cyclohexanone comme agent déshydratant et éclaircissant en technique histologique. *Bull. d'Histol. Appl.*, 19, 55. 1942.

Cyclohexanone can be used satisfactorily for dehydration and clearing instead of the traditional absolute alcohol followed by xylene or toluene. This saturated cyclic ketone has a number of advantages: a high boiling point ( $140^{\circ}$  C.), a low melting point ( $-40^{\circ}$  C.), a density close to that of water, miscible with organic solvents and paraffin, only slightly inflammable, non-toxic, and does not harden the tissues.

The procedure used is as follows: Fix and wash tissues as usual. Partially dehydrate in 95% alcohol for 12 hr. Dehydrate and clear in two successive baths of cyclohexanone, the first for 4 hr., the second for 2 hr. Impregnate in two baths of paraffin for 2 hr. or less each. This is satisfactory for pieces 3 mm. thick; for larger pieces more time would be required for each step. The same series of solutions could be used for the dehydration of sections after staining.—*Jean E. Conn.*

### DYES AND THEIR BIOLOGICAL USES

**GORDON, J., and WALKER, N.** Features in the Congo red molecule associated with the inactivation of complement. *J. Path. & Bact.*, 57, 451-6. 1945.

The inactivation of complement appears to result from its adsorption on the dye. Dyes of quite varied constitution can inhibit complement provided they are of sufficient molecular size.—*S. H. Hutner.*

**LILLIE, R. D.** Effects of metals on Giemsa stain solutions in fifty per cent glycerol and methanol mixtures. *J. Lab. and Clin. Med.*, 31, 253-6. 1946.

Zinc acts on methylated thionin in glycerol-methanol solutions, but not on thionin, to shift their spectral absorption bands toward the red end of the visual spectrum. The use of tin foil or other foils containing tin, zinc or cadmium as a lining for screw caps for Giemsa stain is thus contraindicated. Copper foils containing appreciable admixtures of zinc or tin also seem undesirable. Paraffined cardboard is the most innocuous and generally satisfactory lining for such caps.—*John T. Myers.*

**WOLFROM, M. L., HARRIS, W. D., JOHNSON, G. F., MAHAN, J. E., MOFFETT, S. M., and WILDI, B.** Osage orange pigments. XI. Complete structures of osajin and pomiferin. *J. Amer. Chem. Soc.*, 68, 406-18. 1946.

The ethylenic linkage located in the  $C_6H_5$  residue of both osajin and pomiferin is conjugate to position 8 of the isoflavone nucleus. The pigments are identical in all respects save for the position of an additional phenolic group in position 8' of pomiferin. Both pigments are mordant dyes for wool and silk, but have no effect on cotton. The use of tin and chromium as mordants gives especially bright colors.—*R. T. Whittenberger.*

### ANIMAL MICROTECHNIC

**McMANUS, J. F. A.** The demonstration of certain fatty substances in paraffin sections. *J. Path. & Bact.*, 58, 93-5. 1946.

The following technic permits the clear demonstration of certain lipids—probably phospholipids—in sections made by the conventional paraffin method. Fix 1 to 5 weeks in the following solution: cobalt sulfate or nitrate, 1 g. in 80 ml. distilled water; 10 ml. of 10%  $CaCl_2$ ; and 10 ml. formalin. If  $CoSO_4$  is used, a precipitate forms, which is covered with cotton and used to support the tissue. If desired post-chrome 24 to 48 hr. in 3%  $K_2Cr_2O_7$ . Take blocks of tissue 2 to 3 mm. thick, blot dry, and dehydrate in 3 changes of acetone,  $\frac{1}{2}$  hr. each. Alcohol

dehydration is also permissible. Place directly in melted paraffin at 55–60° C., change 2 to 3 times in an hour, then block. Cut sections 4 to 6  $\mu$ , fix to slides, pass through xylene and 70% alcohol. Stain 30 min. at room temperature in a saturated solution of Sudan black in 70% alcohol. Rinse in 50% alcohol and mount in glycerol. Lipids are deep blue-black.

Material thus stained with Sudan black does not stain with osmic acid or Sudan IV. Myelin is stained. Mitochondria in kidney are clearly demonstrated.—S. H. Hutner.

**SCHAIN, P.** Cytoplasmic cilia-like extrusions of macrophages as demonstrated by a modified Wright's stain. *Amer. J. Clin. Path.*, 15, (Tech. Sect. 9.), 49. 1945.

In pleural and peritoneal fluids cells of reticuloendothelial origin were observed which showed hair-like appendages, resembling cilia. These cilia-like projections from the cytoplasm of the cells were only demonstrable by staining the smears with a modified Wright's stain, or in supravital preparations of freshly removed living cells stained with neutral red and diazine green. The modification of Wright's staining procedure consists of using a diluting fluid buffered to pH 8 instead of 6.8 and of staining at least 10 min. instead of 3 min. The relation of these cytoplasmic extrusions of the reticuloendothelial cells to the formation of normal and immune globulins in the body is briefly discussed.—L. Farber.

**TILDEN, I. L., and TANAKA, M.** Fite's fuchsin-formaldehyde method for acid-fast bacilli applied to frozen sections. *Amer. J. Clin. Path.*, 15, (Tech. Sect. 9), 95. 1945.

A rapid acid-fast staining technic for frozen sections is described which yields suitably stained slides in a much shorter time. The technic recommended is as follows: Fix tissue 24 hr. in 10% formalin or 1:1 mixture of 10% formalin and 95% ethanol; cut frozen sections 10–15  $\mu$  thick; affix to slide, let stand to near dryness, cover with 95% ethanol, blot and treat 1 min. with absolute ethanol; drain slide, flood with thin celloidin (0.9% solution of dry celloidin in 1:1 ether and absolute ethanol) wipe off excess celloidin around section; before drying is complete place at least 15 min. in carbol fuchsin at room temperature (0.5 g. Magenta III, 5.0 g. phenol crystals, 10 ml. methanol or ethanol, made up to 100 ml. with distilled water); wash, and place 5 min. in 40% neutral reagent grade formalin (the section turns blue); wash, and treat 5 min. with acid alcohol (1% HCl in 70% ethanol); wash, and treat 2–5 min. with 1% aqueous  $\text{KMnO}_4$  (section turns brown); wash and treat with 2% aqueous oxalic acid 30 sec. or until just a trace of brown color remains; wash and stain with Harris hematoxylin, 2–5 min.; wash, stain with a modified Van Gieson's mixture (acid fuchsin 0.01 g., picric acid 0.5 g., distilled water to 100 ml.), 3 min.; treat with 95% ethanol, 2 min., blot; add 99% ethanol or isopropanol, 1 min., blot; clear in 2 changes of xylene 5–10 min. each; mount in gum damar.

With this technic, nuclei appear brown, muscle yellow, collagenous connective tissue red, acid-fast bacilli deep ultramarine blue and non-acid-fast organisms are unstained.—L. Farber.

**WACHTENSTEIN, M.** Alkaline phosphatase activity in normal and abnormal human blood and bone marrow cells. *J. Lab. & Clin. Med.*, 31, 1–17. 1946.

The technic is a modification of Gomori's method for visualizing alkaline phosphatase activity, as follows:

Air-dry blood or bone marrow films as usual. Fix with a solution containing 0.25 g. celloidin in 50 ml. absolute alcohol and 50 ml. ether. Dip in 95% alcohol. Wash in distilled water and place for 15 hr. at 37°C. in a solution consisting of: 3.2% aqueous sodium glycerophosphate (+5  $\text{H}_2\text{O}$ ), 10 ml.; 10% aqueous sodium diethyl barbiturate, 10 ml.;  $M/10$   $\text{MgSO}_4$ , 10 ml.; 2% aqueous  $\text{Ca}(\text{NO}_3)_2$ , 5 ml.; distilled water, 50 ml.

Wash in water containing a few drops of the calcium nitrate solution, put in a solution of 2% cobalt nitrate for 5 minutes, wash in tap water, and place in a Coplin jar with water containing a few drops of ammonium sulfide. The tissue phosphatase liberates inorganic phosphate from the substrate, and it is precipitated by the calcium ions in the incubating solution. The inorganic phosphate becomes cobalt phosphate, which then becomes black cobalt sulfide. The

number of pus cells as well as the intensity of the phosphatase staining increases with extent of infection, probably due to increased metabolic activity. This activity was markedly decreased in acute myeloid leukemia.—*John T. Myers.*

**WEBER, A.** *Recherches sur la fixation des tissus adultes ou embryonnaires en vue de l'imprégnation des éléments nerveux par les sels d'argent.* *Bull. d'Histol. Appl.*, 19, 129-36. 1942.

The author has developed a method of fixation which makes possible excellent silver impregnation of even the finest nerve fibers. His procedure is to fix with formaldehyde in a slightly acid medium for several days. Then the tissues are washed in a mixture of dioxane and propyl alcohol to complete the extraction of lipids. After a rather rapid washing in distilled water, they are ready for impregnation by Bielschowsky's technic. He has succeeded in demonstrating nerve fibers in a variety of tissues including chicken embryos and the brains of fish and of adult rats.—*Jean E. Conn.*

**WHITE, B. M., and CULBERTSON, C. G.** A modified Gram's stain for use in staining large numbers of smears. *Amer. J. Clin. Path.*, 15, (Tech. Sect. 9), 53. 1945.

A Gram-staining method is described for routine use on large numbers of smears of varying densities which gives better differentiation than other procedures tried. The main difference is the use of potassium mercuric iodide as a mordant. The recommended technic is as follows: Stain at least 1 min. with a 1% aqueous filtered crystal violet solution, adding 5 drops of 5% aqueous  $\text{NaHCO}_3$  solution to each slide; drain and dry by a hot air stream and treat at least 1 min. with 1% aqueous potassium mercuric iodide solution (made by dissolving two potassium mercuric iodide tablets, obtained from Davis and Geck, Inc., 57 Willoughby St., Brooklyn, N. Y. in 100 ml. of distilled water), or just wash off stain with a 1% aqueous potassium mercuric iodide solution and let stand 1 min. or more with the solution; wash with tap water; decolorize with acetone until no more color remains; wash with tap water; counterstain with 0.1% safranin O or Pappenheim's pyronin-methyl-green; wash with tap water and dry.

It is important that the crystal violet should not be washed off with water before the addition of the potassium mercuric iodide. The authors state that the use of this method has eliminated false findings and that a striking differentiation of Gram positive and Gram negative organisms is obtained.—*L. Farber.*

### PLANT MICROTECHNIC

**WHITTENBERGER, R. T., and BRICE, B. A.** Rapid estimation of rubber in guayule latex dispersions. *Ind. Eng. Chem., Anal. Ed.*, 18, 209-10. 1946.

A microscopic method for the rapid estimation of rubber in crude guayule latices is described. The latex is diluted to give 2 to 5 rubber particles per square of a Petroff-Hausser bacteria counter. For one determination, all rubber particles, irrespective of size, are counted in 36 squares in each of two mounts. The spherical rubber particles can be distinguished readily from the non-rubber particles such as chloroplasts, protoplasmic granules, cell wall fragments, bacteria and silt. The method is standardized by making counts on a number of guayule latices of known rubber content, as determined by chemical analysis. After standardization, the concentration of rubber (in mg. per 100 ml.) in an unknown dispersion (C) is calculated by the equation  $C = 1.85 \text{ ND}$ , where N is the observed average number of particles per square in the diluted dispersion, and D is the dilution factor. It is suggested that the method may be useful in selection, breeding, and recovery work on other rubber-bearing plants, including hevea.—*R. T. Whittenberger.*

### MICROORGANISMS

**CAMERON, GEORGE M., and CASTLES, RUTH.** Detection of tubercle bacilli in sputum by the clorox method and the direct smear. *J. Lab. & Clin. Med.*, 31, 361-8. 1946.

The direct smear, clorox, and sodium-hydroxide-alum methods for demonstrating tubercle bacilli in sputum were carefully compared. The clorox method

was somewhat more efficient than the NaOH-alum method, giving twice the number of acid fast organisms per field. Both the clorox and the NaOH-alum methods surpass the direct smear. The clorox method is much simpler, gives more complete digestion, and shows less visible extraneous material on the slide. When using the clorox method, a swab should be moistened in clear blood serum before transferring sediment to slides. This provides a background and fixative when only a few acid fast bacilli are present.—*John T. Myers.*

**DIAMOND, L. S.** A new rapid stain technic for intestinal protozoa using tergitol-hematoxylin. *Amer. J. Clin. Path.*, 15, (Tech. Sect. 9), 68. 1945.

The use of the aliphatic penetrant tergitol No. 7 (Carbide and Carbon Chemicals Corp.) is recommended as a substitute for heat to decrease the hematoxylin staining time. The recommended procedure is as follows:

Prepare a uniform smear of a feces emulsion in physiological salt solution, and immediately fix 2 min. in Schaudinn's solution, to which 5% glacial acetic acid has been added just before use; treat 1 min. with 50% ethanol; wash 1 min. in water; mordant 5 min. in 4% ferric ammonium sulfate solution; rinse quickly in distilled water; stain 5 min. in Hance's (Science, 77, 287, 1933) tergitol-hematoxylin solution (to a 5% hematoxylin solution in 95% ethanol diluted to 0.5% with distilled water add a "minute quantity" of sodium bicarbonate to ripen and to every 30–40 ml. of this solution add 1 drop of tergitol No. 7 just before use); rinse in distilled water; destain 3–5 min. in saturated aqueous picric acid; rinse in 2 changes of distilled water; treat with potassium acetate solution (the salt added to distilled water until the pH is above 7.0) until blue; treat 1 min. each with 70% and 95% ethanol, and 2 min with absolute alcohol or carbol-xylene; treat twice for 1 min. each time with xylene; mount in balsam, gum damar or clarite.

The smears should never be allowed to dry. For more permanent or special study preparations it is recommended that after fixation the slides be washed a few minutes in an iodine-alcohol solution to remove all mercuric chloride, and that a more gradual alcohol series be used to insure complete dehydration. The staining procedure gives results comparable to those with Heidenhain's iron-hematoxylin method but in a shorter time.—*L. Farber.*

**FELSENFELD, O., and YOUNG, V. M.** An improved method for the examination of intestinal protozoa. *Amer. J. Clin. Path.*, 15, (Tech. Sect. 9), 47. 1945.

A hematoxylin staining technic is described for use with suitably fixed smears of fresh fecal samples. The fixing solution, a modified Schaudinn's fluid, has the following composition: 200 ml. saturated aqueous  $\text{HgCl}_2$ ; 100 ml. 96% ethanol (before use 7 ml. of glacial acetic acid are added to each 100 ml. of this mixture). Slides are delivered to the laboratory in this solution and may be left in it up to 48 hours. The slides are then treated as follows: Immerse 5–10 min. in 96% ethanol containing enough iodine to give a "rich port wine color;" immerse 5 min. in 70% ethanol (if staining cannot be completed the same day slides may be left in 70% ethanol overnight); stain at least 30 min. in Mallory's phosphotungstic-hematoxylin solution (dissolve 0.1 g. hematoxylin for "histologic staining" in 50 ml. of boiling distilled water; when cool mix with 20 ml. 10% aqueous C. P. phosphotungstic acid and make up to 100 ml. with distilled water; add 10 ml. freshly prepared 0.25% aqueous potassium permanganate); immerse slides 5 min. or until smears turn deep blue in a Coplin jar containing distilled water; treat 5 min. each with 70%, 95% and absolute ethanol and xylene; mount with clarite and cover with cover slip.

The smears should not be allowed to dry during the entire procedure.—*L. Farber.*

**FUENTES, CESAR.** A method for differentiating *Candida albicans* in tissue. *J. Bact.*, 51, 245–6. 1946.

The fat drops in *Candida albicans* can be stained with fat-soluble dyes and the parasites thus clearly demonstrated in tissue sections. The following method results in much less distortion of both blastospores and hyphae than the methods using alcoholic solutions of the dyes: Stain the thinnest possible frozen sections 5 min. in 1% fresh, filtered Sudan IV in pure (reagent grade?) lactic acid; wash

in distilled water; place sections in 1% aqueous toluidine blue for 2 min.; wash carefully; mount in Apathy's fluid or in a saturated glucose solution. The method was equally effective with fresh tissues or those preserved in 10% formalin for as long as a year.—*Virgine Kavanagh.*

**RITTERHOFF, R. J., and BOWMAN, M. G.** Demonstration of tubercle bacilli by fluorescence microscopy. *Amer. J. Clin. Path.*, 15, (Tech. Sect. 9), 39. 1945

A comparative study was made of the demonstration of tubercle bacilli by the Ziehl-Neelsen staining method, the fluorescence microscopy procedure, and by animal inoculation.

The methods employed were as follows: Spencer Lens Company fluorescence microscopy accessories were used. For most of the work a 4 mm. objective and a 10× ocular were used, giving a 450 diameter magnification. Later an 8 mm. objective and a 20× ocular were substituted giving a somewhat lower magnification, 400 diameter, but a greater brilliancy of fluorescing organisms. Smears were fixed to slides with egg albumin, air dried and flamed. Slides were stained 2-5 minutes in 0.1% auramine O in 3% phenol, decolorized twice for 2-5 min. with 0.5% HCl in 70% alcohol. No counterstain was used. The tubercle bacilli appeared brilliant yellow on a nearly black background. The faintly fluorescent egg albumin aided in focusing. The Ziehl-Neelsen staining procedure employed was that of "institution 14" in Pottenger's review of staining procedures (*Amer. Rev. Tuberc.*, 45, 549, 1942). Sputa, gastric contents, feces, urines and spinal fluids were concentrated by the methods of Hanks, Clark & Feldman, (*J. Lab. and Clin. Med.*, 23, 736, 1938; 25, 974, 886, 1940). Pleural and ascitic fluids and exudates were centrifuged without previous treatment. Of 2918 specimens examined, 50.5% were Ziehl-Neelsen positive and 60.6% were fluorescent positive. Of these specimens, for example, 11 showed 2 or more fluorescent positive, and Ziehl-Neelsen negative sputa, and 36 patients had earlier sputa positive by fluorescence only and subsequently positive by both staining methods.

The specificity of the fluorescent method was determined by guinea pig inoculations; 106 animals were inoculated with material from 102 cases that were fluorescent-positive but Ziehl-Neelsen-negative. Nine positive tuberculous animals were found, or 8.4%.

The above results indicate that the fluorescence microscopy technic closely approximates the most sensitive methods for demonstrating tubercle bacilli (H. C. Sweany, *Amer. J. Clin. Path.*, 12, 458, 1942). The specificity of the method does not equal its sensitivity, as evidenced by the low incidence of positive tuberculous animals resulting from inoculation with material that was fluorescence-positive but was Ziehl-Neelsen-negative. The authors conclude that the above results "would obviate the routine use of the fluorescence method because of failure to correspond with the clinical, roentgenographic or pathologic status of the tuberculous patient, and, more importantly, because of the lack of specificity as observed in this investigation."—*L. Farber.*

**WELLS, WILLIAM F., WINSLOW, C.-E. A., and ROBERTSON, ELIZABETH C.** Bacteriological procedures in the evaluation of methods for control of air-borne infection. *Amer. J. Pub. Health*, 36, 324-31. 1946.

Nasopharyngeal parasites—specifically hemolytic streptococci of the nose and throat—can be isolated in media containing proteose and tryptose peptones. Blood agar containing these ingredients becomes selective for the organisms upon the addition of 2 ppm. of gentian (crystal?) violet, which inhibits most common staphylococci in air. Organisms enriched in proteose-tryptose broth, containing lactose and brom thymol blue to indicate acid production, and ¼ ppm. of gentian violet to inhibit staphylococci, may be transferred to the selective medium for confirmation. Tubes showing acid in 24 or 48 hours are streaked on the gentian violet blood agar, hemolytic organisms being further confirmed as Gram-positive.—*M. W. Jennison.*

# STAIN TECHNOLOGY

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## THE PICCOLYTE RESINS AS MICROSCOPIC MOUNTING MEDIA

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**ABSTRACT.**—A new series of synthetic resins, the "Piccolytes" ( $\beta$ -pinene polymers), is recommended for permanent mounting media in histological work. These resins, which are available with various melting points, are of correct refractive indices, very low acid numbers, are pale, non-yellowing, have good adhesion to glass, and are freely soluble in xylene. Of a considerable variety of newer synthetic resins which were tried (stimulated by the recent scarcity of Canada balsam), only these terpene resins were found entirely satisfactory. Being of controlled manufacture, they are uniform in characteristics as well as readily available and very cheap.

A partial review of the literature of synthetic resin mountants is included.

### INTRODUCTION

There are only a few good permanent mounting media suitable for histological work, and these are almost exclusively resins, natural or synthetic. The *possible* failings of such widely used natural resins as Canada balsam, gum damar, and gum sandarac are well known. Some preparations are quite satisfactory, but others tend to turn yellow, to crack with age, or to develop acidity that affects basic stains. On the other hand, Davies (1940) found many "neutral balsam" samples actually alkaline enough to fade acidic stains. Shillaber (1944) tells of slides mounted with balsam eighty years ago and still in good condition, and yet all workers have seen balsam slides that began to yellow, crack or fade in a much shorter time. The real criticism of the natural resins is that, like all complex natural products, they are variable in composition and consequently unpredictable in behavior. Here is the obvious advantage of *synthetic* resins produced by controlled manufacture, *provided* the desired microscopical characteristics can be

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obtained. Let us briefly tabulate these requirements, borrowing in part from Cowdry (1943), Shillaber (1944), and Chamot and Mason (1938):

- 1.) Correct refractive index for resolution of stained cellular elements (balsam,  $n \approx 1.53$ )
- 2.) Freedom from acidity that would fade basic dyes
- 3.) Clarity: colorless or quite pale, and should not yellow with light or age
- 4.) Solubility in a suitable medium, such as xylene (or toluene) for the ordinary technic
- 5.) Good adhesive properties for glass
- 6.) Drying: should not require too long to harden, and should remain amorphous and not crack or granulate
- 7.) Should be constant in composition, stable, inert, easily available and not too expensive
- 8.) Should not have too low a softening point for slides used in projection.

#### PRESENT SYNTHETIC MOUNTANTS

At present, the best and most familiar synthetic resin mountant is "Clarite X" ( $n = 1.567$ ), a naphthalene polymer made by the Neville Company of Pittsburgh. "Clarite" ( $n = 1.544$ ), a very similar material, appears no longer available. Clarite X is water-white, of very low acidity and fair adhesion to glass. It is usually employed in 60–70% toluene solution, although some workers, such as Davies, prefer to use xylene—in which it is readily soluble. The Clarites are generally reported to yield excellent results, although we have heard of Clarite slides which developed peripheral fading or partial separation of the cover slip after two or three years. Mohr and Wehrle (1940) have stated that Clarite-mounted slides tend to develop air bubbles by unequal contraction. Clarite is the newer name for "Nevillite V" ( $n = 1.544$ ), a cycloparaffin resin suggested by Groat (1939), together with "Nevillite I" ( $n = 1.567$ ), both of which are apparently no longer sold under these names. Groat reported good results with no undesirable features noted other than a slight yellowing slowly developed by Nevillite I (Clarite X). A preparation put out by the Fisher Scientific Company in toluene solution under the name of "Permout" is a naphthalene polymer, doubtless identical with Clarite X. These naphthalene resins have much higher softening points (approximately  $150^{\circ}\text{C}.$ ) than the natural products. This is advantageous for slides intended for projection, although the plasticity is probably less.

## OTHER SYNTHETICS PROPOSED

A number of less satisfactory transparent synthetic plastics or resins has been suggested, of course. Preston (1930) proposed nitrocellulose for mounting "unstained cellulose materials". Hanna (1930) recommended "Hydrax", a naphthalene resin of high refractive index ( $n = 1.822$ ) for diatoms. Fleming (1943) himself synthesized another polynaphthalene resin of high refractive index ( $n = 1.80$ ) which he described and patented, naming it "Naphrax". It was also intended for diatoms and is not suitable for tissues. Still another high refractive index resin is one of the "Aroclors" ( $n = 1.63$ ), chlorinated diphenyl polymers manufactured by Monsanto Chemical Co. and mentioned by Shillaber.

Polystyrene (under the name of "Distrene") in xylene, plasticized with tricresyl phosphate, was proposed by Kirkpatrick and Lendrum (1939) and again later by Hutner (1941). With dibutyl phthalate, a superior plasticizing agent, it was again suggested by the former (1941). In our own experiments, we found polystyrene (Dow Chemical Co.) to give fair results. The polymer is water-white and of low acidity, but the refractive index is slightly high ( $n = 1.59-1.61$ ). The adhesion to glass when using polystyrene in xylene is very poor. However, the addition of 5-10% of dibutyl phthalate gives great improvement.

Naturally, the new methacrylate resins, methyl, ethyl and isobutyl polymers, which have been used for embedding whole specimens and as "substitutes" for cover slips, have also been proposed as microscopic mounting media. Skiles and Georgi (1937) advocated "Pontalite" (now "Lucite", du Pont's name for the methyl methacrylate plastic) only for permanent mounts for bacteria and molds, but Cole (1938) recommended a dioxane solution of the polymer wherever balsam or damar would be used. Richards and Smith (1938) protested that Lucite decolorizes the stained specimen, clears poorly and forms bubbles in drying. Davies (1940), using a methyl methacrylate polymer called "Diakon" ( $n = 1.503$ ) in xylene, had no difficulty with fading of stains, but the cover slip loosened seriously. We have tried solutions of "Plexiglas" (Rohm and Haas' name for the same material) and found it worthless. At the time when glass cover slips were becoming a scarce item, O'Brien and Hance (1940) suggested that a solution of polymerized isobutyl methacrylate in xylene or benzene be allowed to form a film of the plastic over the specimen. As a result, Central Scientific Co. (1940) advertised the polymer as a substitute for Canada balsam. Groat (1940) promptly declared it highly unsatisfactory for tissue

work, pointing out that the refractive index is too low ( $n = 1.47$ ), it has poor adhesion for glass, it fades certain stains, and possesses too low a softening point. Shillaber, while citing Groat, nevertheless feels that for temporary mounts intended for photomicrography, a solution of isobutyl methacrylate polymer has its uses.

### THE PICCOLYTE RESINS

When the supply of Canada balsam became uncertain, we developed an interest in testing newer pale synthetic resins as possible substitutes, seeking a xylene-soluble, low-acidity, abundant material. Quite a number of synthetic resins of various chemical types were tried in a variety of solvents. Slides were made, and then with the more promising mountants, refractive indices and acid numbers (mg. KOH to neutralize free acid per gram of resin) were determined. Many resins were worthless, usually because of incorrect refractive index or of excessive acidity, a few were fair, and one series of resins, the "Piccolytes", proved excellent.

TABLE 1. AVERAGE VALUES OF REFRACTIVE INDICES AND ACID NUMBERS AS EXPERIMENTALLY DETERMINED

Piccolyte Resin	Refractive Index(20° C.)	Acid Number
S-70	1.524	0.36
S-85	1.529	0.40
WW-85	1.528	0.54
S-100	1.528	2.08
S-115	1.531	0.49

The Piccolyte<sup>1</sup> resins are new synthetic thermoplastic terpene resins, being  $\beta$ -pinene polymers. The usual standard series resins (designated by an "S" and followed by a number which is the approximate softening point in degrees C.) are listed in a variety of melting points—from about 10°C. ("S-10") to 125°C. ("S-125"). The lower ones are viscid liquids or semi-solids, the higher ones pale yellow resins. (S-125 was not available at the time of our experiments.) The Piccolyte resins are all of very light color, of uniform composition and freely soluble in xylene, toluene and other hydrocarbon solvents. They also dissolve in chlorinated hydrocarbons such as chloroform but do not give a satisfactory solution in dioxane. These terpene resins meet all the requirements which we tabulated earlier. The manufacturer claims that the Piccolytes are "non-yellowing" and that the acid number is "less than four". Our

<sup>1</sup>Manufactured by the Pennsylvania Industrial Chemical Corp., Clairton, Penn. We are very grateful to Messrs. F. W. Corkery and J. W. Church for supplying both generous samples and information.

many titrations always found it much lower than that. There is also a "WW" or "water-white" series (hydrogenated  $\beta$ -pinene polymers) of which we were able to obtain only "WW-85" at the time of our experiments.<sup>2</sup> This series should be of still lower acidity. The price of all Piccolytes is very trivial, being listed at 15½-18½ cents a pound. Our samples were received gratis. The variety of resins available with regard to softening point is also of value if projection is considered. The refractive indices were measured by melting the resin directly upon the main prism of an Abbé refractometer (West, 1938).

Slides were prepared of a variety of tissues of mouse and rat, stained with hematoxylin-eosin and mounted with xylene or toluene solutions of the above Piccolyte resins. No trouble was encountered with trapped air bubbles, and the adhesion to glass was good without added plasticizer. The definition was very satisfactory, all slides being examined from lower powers to a thousand diameters (with oil). Some of these slides are now over two years old, and as yet there has been no suggestion of yellowing, granulation, or fading. No differences could be observed between the various Piccolytes, all being very similar except for the softening temperature. Control slides were mounted with Clarite X in toluene.

Theoretically, the WW series should be slightly more desirable than the S series, as the former are intended to be even paler and of even lower acid number.

For slides intended to be projected, S-70 is of rather low softening point. Here, those resins melting at 115° or 125° (when available) are doubtless preferable. However, on long ageing, unless a plasticizer is present, the higher softening resins may have a greater tendency to develop irregularities.

For ordinary purposes, we recommend WW-85, S-85, WW-100 or S-100 — as obtainable. There are individual preferences in the thickness of the mounting solution of course. We found satisfactory a solution where the weight of the resin in grams is 1.5 to 1.7 times that of the volume of xylene in cubic centimeters. For example, break up the resin into small lumps (if it is powdered, it will simply cement together again), weigh out 30-34 grams, place in a 2 ounce *screw cap* bottle and add 20 ml. of pure xylene. Let stand in a warm place for a couple of days with occasional shaking.

All of the Piccolytes fluoresce under ultra-violet light, as do practically all of the permanent mountants.

<sup>2</sup>WW-100 and WW-115 are now also available, but S-125 is still out of production.

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## ROOT TIP SMEARS FOR MAIZE

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Frequently it is necessary to have counts of root tip chromosomes, but the paraffin method for making preparations is laborious and time consuming. However, division figures equal in excellence to those from sectioned material can be obtained quickly and easily by the following technic (Fig. 1, 2, 3): Fix young actively growing root tips in Carnoy's fluid for 6-24 hours. Change to 70% ethyl alcohol. The material can be kept here until it is convenient to make the preparations. Transfer to equal parts of hydrochloric acid and 95% ethyl alcohol for five minutes, then to 70% ethyl alcohol

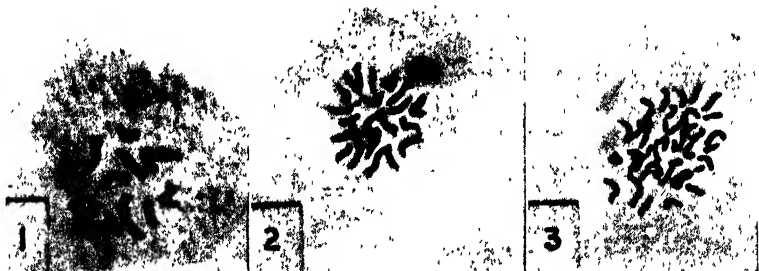


FIG. 1. Haploid root tip (10 chromosomes) 950 $\times$ .

FIG. 2. Diploid root tip (20 chromosomes) 950 $\times$ .

FIG. 3. Tetraploid root tip (40 chromosomes) 950 $\times$

for five minutes. Put a thin free-hand cross-section slice of the root tip into a drop of aceto-carmine on a slide, and tease the material apart with steel needles, or flatten it with a scalpel. Put on a clean cover glass, and press gently with the eraser end of a pencil. Heat the slide several times by passing through a flame. Examine to see whether there are sufficient division figures. If not, make a preparation from a different section of the root, or from a different root. A good preparation has the cells well separated but intact, with many well-stained division figures. Temporary mounts can be sealed with a gum-mastic paraffin mixture and kept in a cool place for several weeks; or the slides may be made permanent by McClintock's method (1929) for making sporocyte smears permanent.

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## **SMEAR METHODS FOR THE STUDY OF CHROMOSOMES IN ASCOMYCETES**

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**ABSTRACT.**—Several modifications of the aceto-carminc smear technic used by the author in studies of the meiotic chromosomes of *Neurospora* are outlined. The critical role of the staining solution is stressed and suggestions are given for preparing a satisfactory carmine solution. The ease and rapidity of this method suggests its use in cytologic work on other Ascomycetes.

### **INTRODUCTION**

The popular aceto-carminc and aceto-orcein smear technics offer several advantages over other cytological procedures in the study of the meiotic chromosomes of *Neurospora* and other Ascomycetes. The minute tangled chromosomes developed in the ascus divisions are easily studied in smear preparations but often prove difficult to interpret when sectioned. The selective action of carmine and orcein readily differentiates chromatin from other inclusions in the ascus, a feature not true of all nuclear dyes. In cytogenetic studies the rapidity of these smear methods is of prime importance. Several modifications of these technics which have proved useful in recent studies on *Neurospora* are outlined below.

### **PROCEDURE<sup>1</sup>**

1. *Fixation.* Perithecia from cultures grown on Difco cornmeal agar medium at room temperature will usually contain asci in all stages of development 7–10 days after inoculation of the cultures. Blocks of agar containing perithecia are cut out and immersed in 1 part commercial propionic acid and 3 parts 95% ethyl alcohol. Removal of air from the perithecia by aspiration will hasten fixation, but the pressure changes may cause the delicate asci to collapse. Better results are obtained when fixation is accomplished without aspiration. Immersion for 24–36 hours in the fixing solution insures adequate penetration of the ascus mass, and the perithecia

<sup>1</sup>The author wishes to acknowledge several suggestions concerning the preparation of the staining and fixing solutions made by Professor A. O. Dahl and Miss Agnes Hansen of the Botany Dept., Univ. of Minnesota, and also the loan by them of a well ripened sample of aceto-carminc.

may remain in the fixative for a week or longer without harm. With older perithecia a longer period of fixation is helpful in removing much of the oily material accumulated in and around the developing asci.

2. *Smearing*. Fixed perithecia are transferred individually with fine curved dissecting needles to a drop of staining solution on a clean dry slide. Very slight pressure on the wall of the perithecium with the tip of the needle will force the ascus mass through the ostiole. Overfixed or damaged perithecia may be detected at this time for the contents of these will not be forcibly ejected from the ostiole under pressure and the perithecium will seem flaccid. After the asci are forced out, the perithecial walls should be picked out of the stain and discarded since these walls are heavy and rigid and tend to support the cover slip and interfere with smearing. The ascus masses may be oriented in any way, or they may be dissected so that individual asci are separated, before a cover slip is placed on the preparation. When dealing with older material a slight pressure on the cover slip is sometimes necessary to spread the asci apart, but ordinarily its weight is sufficient to provide satisfactory smearing.

3. *Staining*. Staining may be hastened by gently heating the slide over a flame or warm plate. However, much better preparations are obtained by placing the smeared slides overnight in a refrigerator at 3°C. This treatment slows the accumulation of stain in the cytoplasm and thus eliminates the tendency of the asci to overstain. At 3°C. optimum staining is usually obtained 24–36 hours after smearing. In preparations of very young asci the staining is more rapid and best results are observed 12–15 hours after staining. Slides held at room temperature are adequately stained in 3–6 hours, or sooner if heated. For most preparations aceto-carmine proves quite satisfactory but occasionally aceto-orcein may be more desirable. Aceto-orcein is particularly useful in those cases where the large nucleolus obscures some of the chromosomes, since it does not react with the nucleolus. If it later becomes desirable to study the nucleolus McClintock's<sup>2</sup> method of running aceto-carmine under the cover slip of the orcein stained preparation works well.

Slides sealed with paraffin and placed in the refrigerator may be kept satisfactorily for several days. To date the author has not had great success in rendering these slides permanent by the usual methods, since the asci do not stick tenaciously to the slide and are

<sup>2</sup>McClintock, B. *Neurospora*. I. Preliminary observations on the chromosomes of *N. crassa*. *Amer. J. Bot.*, 32, 671–8. 1945.

usually washed off during dehydration. This can be overcome by the use of an albumen fixative on the slide prior to smearing but, as would be expected, such preparations lack the brilliance of those prepared without albumen.

The preparation of the staining solution is the most important step in obtaining satisfactory ascal smears. Different lots of solution vary greatly in their staining properties and this variability has not been adequately explained so that at present "trial and error" is the favorite method of procuring a highly selective stain. In general it seems true that the older the stain solution the better it works, and also that the variable results are not inherent in the sample of dye used, but are rather a function of the mixed solution. Several methods have been suggested to hasten ripening of the stain. The author found the following procedure yielded a very good solution although it must be admitted that the results obtained with this mix did not equal in clarity and brilliance the results secured with a solution prepared in the same way and allowed to ripen for two years on the laboratory shelf.

A mixture of 50 ml. glacial acetic acid and 90 ml. absolute alcohol was poured over 3 grams of carmine in a 500 ml. pyrex beaker. This mixture was brought to a boil and simmered very gently over an open flame for 6 hours. It was then allowed to cool, made up to original volume with absolute alcohol and filtered. It was stored in a clear soft glass bottle and was placed uncorked in full sunshine on the laboratory table. At the end of a week it was once more made up to volume with absolute alcohol, and thereafter the bottle was kept stoppered. The same procedure was followed with equally good results in mixing aceto-orcein. The use of steel dissecting needles in manipulating the perithecia in the staining solution prior to smearing introduces sufficient iron salts into the stain to achieve maximum effectiveness.

The methods outlined above have been used primarily for the study of meiotic divisions in the asci. However very excellent mitotic figures are revealed by these procedures in the sterile paraphyses and in the basal cells of the hymenium. In these cells the minute somatic nuclei become expanded and in many instances the chromosomes may be more accurately counted in these positions than in the asci.



## RELIABILITY OF ETHYL ALCOHOL SUBSTITUTES IN PREPARING ROUTINE HOSPITAL BACTERIOLOGICAL STAINS

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**ABSTRACT.**—Three different bacterial stains were prepared using ethyl, isopropyl and methyl alcohols as solvents for the dry stain. The stains thus prepared were tried against various organisms and their staining qualities noted. Stains prepared with methyl alcohol were comparable, in ease of preparation and staining quality, with those prepared using ethyl alcohol. It was concluded that stains prepared with methyl alcohol, instead of ethyl, would be entirely satisfactory for routine procedures. This confirms the findings of Conn and Darrow.<sup>1</sup>

### CHEMICALS

The biological stains used in the preparation of the staining solutions were Commission certified. The methylene blue (Cert. No. CA-24, dye content 86%), the basic fuchsin (Cert. No. CF-30, dye content 94%), and the gentian violet (Cert. No. CB-13, dye content 84%). The ethyl alcohol (95%) was U. S. P. quality, iso-propyl alcohol was "Petrohol" 99% by volume, Standard Alcohol Company, and the methyl alcohol was Reagent, Acetone Free, 99.5%, General Chemical Company.

### ORGANISMS

*Staphylococcus aureus*, *Streptococcus hemolyticus*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Klebsiella pneumoniae* and *Neisseria gonorrhoeae* were used as test organisms, employing cultures of varying ages, i. e., 24 to 96 hours.

### PREPARATION OF STAINS

Loeffler's alkaline methylene blue, Ziehl-Neelsen carbol-fuchsin and Stirling's aniline-gentian-violet were prepared, three solutions of each being made, one with methyl, one with ethyl, and one with iso-propyl alcohol. The Loeffler's and Ziehl's were prepared ac-

<sup>1</sup>Conn, H. J., and Darrow, Mary A. 1945. Substitutes for ethyl alcohol. *Stain Techn.*, 20, 115.

cording to the formula given in Staining Procedures<sup>2</sup> and the Stirling's according to the formula in Clinical Diagnosis by Laboratory Methods, Todd Sanford, 10th Ed.<sup>3</sup> In the first two mentioned stains, the dry stain was ground in a glass mortar with the alcohol specified, then diluted with the other solvent and transferred to an amber bottle. In the preparation of the Stirling's, the gentian violet was ground fine in a glass mortar, weighed and transferred to an amber bottle. The alcohol was added and the contents shaken. The aniline water was then added and the whole thoroughly mixed. All of the solutions prepared were filtered. The stains were from 4-6 weeks old, when used.

### METHODS OF STAINING

(1) *Loeffler's alkaline methylene blue*. Prepared slides of *S. aureus*, *C. diphtheriae* and *E. coli* were stained for 2-3 minutes with each of the three Loeffler solutions. At the conclusion of the staining time, the excess of stain was washed off with tap water. The slides were blotted dry with filter paper and examined. All three of the solutions stained satisfactorily.

(2) *Stirling's aniline gentian violet (using the Gram method)*. Prepared slides of *S. hemolyticus*, *E. coli*, *S. aureus*, *K. pneumoniae* and *N. gonorrhoeae* were stained 2-3 minutes with each of the Stirling's solutions. At the conclusion of the staining time, the excess of stain was washed off with tap water. Gram's iodine solution was added, allowed to remain 2 minutes, then washed off with tap water. The slides were quickly decolorized by dipping them into acetone, then rinsed off in tap water. The slides were covered with 0.5% aqueous safranin, which was allowed to stain for one minute, then washed off with tap water, dried and examined. The stains made with ethyl and methyl alcohols were satisfactory. When the stains were dissolved in iso-propyl alcohol, the slides did not decolorize uniformly. The procedure was repeated, with the exception that acid alcohol (3 vol. HCl, conc., plus 97 vol. alcohol) was used to decolorize. No better results were noted than when acetone was used as the decolorizing agent.

(3) *Ziehl-Neelsen carbol-fuchsin*. Prepared slides of *M. tuberculosis* were stained with each of the carbol fuchsin solutions. The stain was heated until vapors arose from the solution, allowed to cool, then decolorized, using acid alcohol (3 vol. HCl, conc., plus

<sup>2</sup>Conn, H. J., and Darrow, Mary A. 1943-45. Staining Procedures used by the Biological Stain Commission. See p. IIIA-6.

<sup>3</sup>Sanford, Todd. 1943. Clinical Diagnosis by Laboratory Methods, 10th Ed. Blakiston, Philadelphia, Pa. See p. 881.

97 vol. alcohol). In this procedure, the acid alcohol used had been prepared with the same alcohol as was used to make the stain. The slides were counterstained with their respective Loeffler stain, washed in tap water, dried and examined. The staining results were good for the ethyl and methyl stains. The slides decolorized with acid iso-propyl alcohol solution did not decolorize satisfactorily, though the Ziehl's stained very well.

TABLE 1. RESULTS OF THE COMPARISON

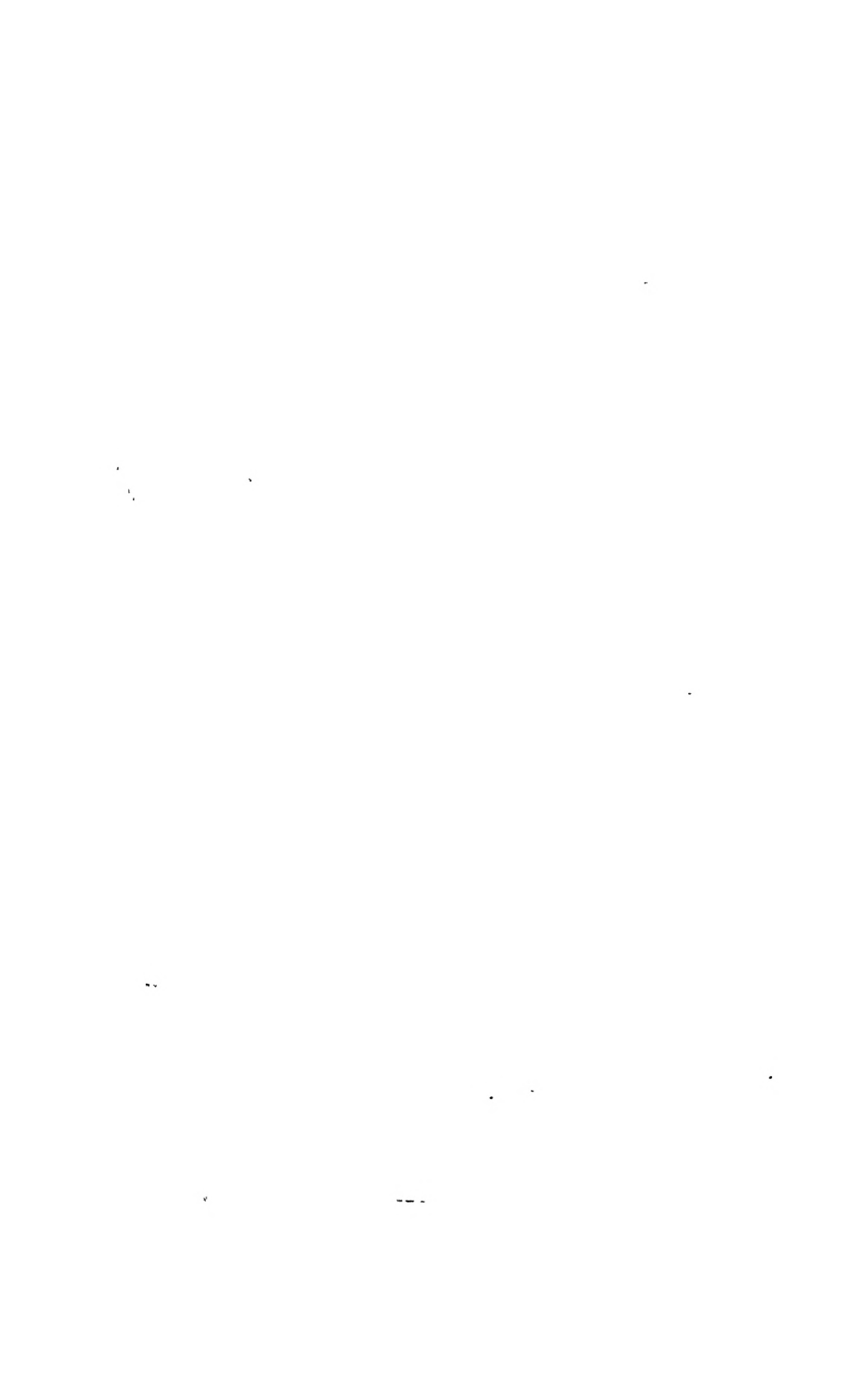
ORGANISM	STAIN PREPARED WITH		
	ETHYL ALCOHOL	PETROHOL	METHYL ALCOHOL
<i>S. aureus</i> . . . . .	S	S	S
<i>S. hemolyticus</i> . . . . .	S	S	S
<i>C. diphtheriae</i> . . . . .	S	S	S
<i>E. coli</i> . . . . .	S	US*	S
<i>M. tuberculosis</i> . . . . .	S	US*	S
<i>K. pneumoniae</i> . . . . .	S	US*	S
<i>N. gonorrhoeae</i> . . . . .	S	US*	S

NOTE: S—Satisfactory US—Unsatisfactory

\*These were considered unsatisfactory because they were not as clear, did not decolorize uniformly or the granules were pale when compared with the slides stained with the ethyl and methyl alcohol stains.

### CONCLUSIONS

From the results obtained (Table 1) using ethyl alcohol and its substitutes, iso-propyl and methyl alcohols, we concur with Conn and Darrow (see footnote 1) that methyl alcohol is the best substitute for ethyl in preparing staining solutions. Due to its low cost and availability, it can be readily substituted in these stains with end staining results fully assured. This substitution should be of interest to all technicians, especially those whose supply of ethyl alcohol is limited or unavailable.



## THE SPECIFICITY OF THE FEULGEN REACTION FOR THYMONUCLEIC ACID<sup>1</sup>

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**ABSTRACT.**—The results of experiments on the specificity of the Feulgen reaction for thymonucleic acid do not substantiate the observations of Carr. The staining is not localized in the nucleus because of the destruction of cytoplasmic constituents following acid hydrolysis or because of the absorbing power of chromatin, since the cytoplasm and nucleolus can still be stained by numerous dyes. The effects of factors such as the acid hydrolysis and sulfurous acid washing baths upon the cytologic distribution of dye were studied on tissues stained with (1) fuchsin-sulfurous-acid (Feulgen) reagent, (2) fuchsin-sulfurous-acid reagent colorized by the addition of formaldehyde, (3) basic fuchsin in one-tenth normal HCl, and (4) basic fuchsin in distilled water. Under comparable conditions, important differences between these stains were found in the effects of preliminary hydrolysis; rapidity of staining and destaining; extractability of dye from tissues by water, alcohol, and sulfurous acid solution; rate of fading from exposure to light; localization of stain in tissues; and differences in hue. After treating tissues with desoxyribonuclease, an enzyme which acts only upon thymonucleic acid, cells do not stain with the Feulgen technic. Following removal of nucleic acid from chromatin by hydrolysis, attempts to demonstrate an absorption of thymonucleic acid upon the residual nuclear protein were unsuccessful.

The evidence for and against the specificity is discussed. In agreement with most other investigators, on the basis of the evidence in the literature as well as these experiments, it is concluded that when properly controlled the Feulgen reaction is relatively specific for thymonucleic acid.

The Feulgen "nuclear" reaction is commonly used as a histochemical test for thymonucleic acid. Any new statements substantiating or discrediting its specificity deserve careful evaluation.

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<sup>2</sup>On leave of absence as advanced medical fellow of the Commonwealth Fund at the Department of Cell Research, Karolinska Institute, Stockholm, Sweden.

If the Feulgen reaction is not selective for staining nucleic acid of the desoxyribose type, the interpretation of much research must be altered and it should no longer be employed as a specific stain. If the claims against the specificity are unjust, the errors should be pointed out promptly, before they are widely accepted by uncritical readers. Certain fallacies in the criticisms of Stedman and Stedman (1943a, 1943b, 1944) and Choudhuri (1943) have already been discussed by Serra (1943), Callan (1943), Barber and Callan (1944), Caspersson (1944), Stowell (1945) and Brachet (1945), and therefore will not be repeated in detail. Recently Carr (1945), another investigator from the same institution as the Stedmans and Choudhuri, has claimed on the basis of (1) color of the stain, (2) effect of hydrolysis and (3) staining of chromosomes, that "nucleic acid is not necessarily concerned in the reaction."

This paper will describe the results of additional experiments initiated following the statements of Carr. The observations on 1,875 sections of tissues in 17 experiments can only be summarized briefly. Milovidov in 1938 compiled 450 references in which the Feulgen technic had been employed and it is now possible that such a list might approximate 1,000. No attempt will be made to review such an extensive literature, but some of the significant evidence for, as well as claims against, the specificity of the Feulgen reaction will be summarized.

#### EXPERIMENTAL OBSERVATIONS

By visual observations, Carr (1945) claims that the bright red stain of basic fuchsin may be converted to the mauve color of the Feulgen reaction by the establishment of comparable pH. In our experiments, however, the addition of HCl to solutions of basic fuchsin or the washing of sections stained with basic fuchsin in N/10 HCl causes a change in color, but the resultant hue is different from that of the positive Schiff test or Feulgen reaction. Carefully controlled spectrophotometric analyses by Stowell and Albers (1943), in which the absorption spectra of basic fuchsin and of fuchsin-sulfurous-acid (Feulgen) reagent containing formaldehyde were measured in equivalent amounts of HCl, showed distinct differences in absorption maxima and absorption coefficients. In agreement with numerous other investigators, it was concluded that the addition compound of fuchsin-sulfurous-acid and aldehyde contains chromophore groups different from basic fuchsin and comparable to those in sections stained by the Feulgen reaction.

Carr suggests that the acid hydrolysis destroys the cytoplasmic structures so that the Feulgen stain is consequently localized within

the nucleus. Although strong acid may destroy cytoplasmic structures such as mitochondria, under the conditions of the Feulgen technic the hydrolysis leaves some cytoplasmic material which stains with such dyes as acid fuchsin, basic fuchsin, pyronin, crystal violet, methylene blue, hematoxylin, and eosin. In several series of experiments a variety of normal and neoplastic tissues of the mouse, rat and man were fixed in Zenker-acetic, Zenker-formol, sublimate-alcohol, Bouin's fluid, formaldehyde and acetone, and paraffin sections were stained with the above dyes. Some sections were hydrolyzed for five minutes at 60°C. in normal HCl as in the usual Feulgen technic, others for twenty minutes at 50°C. as employed in histochemical studies on tumors (Stowell, 1946), and other comparable control sections were stained without hydrolysis. The results of the Feulgen reaction and other stains after the two methods of hydrolysis were comparable. Tertiary butyl alcohol (Stowell, 1942a, 1942b) was employed in the destaining and dehydration of sections stained in pyronin and methylene blue, since it extracts the dye more slowly and permits a more distinct differentiation. After hydrolysis greater differences were noted with tissues preserved in different fixatives than after staining with different dyes. In many tissues, especially in those fixed in poor protein precipitants such as acetone, there was a reduction in the cytoplasmic staining after acid hydrolysis. However, not only was some cytoplasmic material stained by these dyes—which is not stained by the Feulgen reaction—but the nucleolus was stained, and it is generally unstained by the Feulgen technic.

If the staining of the chromosomes by the Feulgen reaction were only a non-specific adsorption phenomenon, as suggested by Carr, the nucleolus, which does not contain appreciable desoxyribose nucleic acid and which adsorbs many other dyes, should stain by the Feulgen technic. Generalizations on the staining of nucleoli should be undertaken cautiously, since the tinctorial reactions depend not only upon variations in nucleolar constituents such as protein and ribose nucleic acid and the state of preservation of these substances, but especially upon the stain and conditions of its application. In sections stained by the Feulgen reaction, there is a concentration of Feulgen-positive material about the periphery of the nucleolar masses, but the central areas are unstained. Under lower magnification the nucleoli appear to be stained because of the intense stain at their periphery. Small nucleoli, which lie entirely within the plane of the section, may appear stained because only the Feulgen-positive chromatin at the periphery is visible.

With the previously mentioned dyes as well as with the modifications of the Feulgen technic mentioned in the next paragraph, the staining of nucleoli is more variable. Although in low concentrations the acidified basic fuchsin and colorized fuchsin-sulfurous-acid may fail to stain the center of the nucleoli, it is stained heavily in less dilute solutions of the dyes.

Other experiments were undertaken to study the relation of various staining procedures with acid and basic fuchsin to the results obtained with the Feulgen reaction. As suggested improved technics to give results comparable to those of Feulgen, Choudhuri (1943) recommends fuchsin-sulfurous-acid (Feulgen) reagent colorized by the addition of aldehyde, and Carr (1945) employs acidified basic fuchsin stain on tissues hydrolyzed by acid. Therefore, tissues fixed in various preservatives were stained with (1) fuchsin-sulfurous-acid (Feulgen) reagent, (2) fuchsin-sulfurous reagent colorized by the addition of formaldehyde, (3) acidified basic fuchsin in one-tenth normal HCl, and (4) basic fuchsin in distilled water. In each instance, the initial concentration of the basic fuchsin was 0.5%. This dye was obtained from the National Aniline & Chemical Company, Inc., had certification No. NF-33 and an 88% dye content. The hydrogen-ion concentration of the first three solutions was the same. Before staining some sections were hydrolyzed in normal HCl and after staining some were washed in the sulfurous acid solutions as in the Feulgen technic. Uniform results were obtained with the Feulgen technic which could only be approximated by the other methods under special conditions, and additional definite differences in the four stains were demonstrated. The acid digestion is essential for the Feulgen reaction, but has less effect upon the others than the sulfurous acid wash, which can extract large amounts of these other stains. The other dyes stain tissues more rapidly, stain the cytoplasm, and are more readily washed out of sections by water and alcohol. Under properly controlled conditions with the Feulgen technic only traces of pigment are ever present in the cytoplasm and they are rapidly removed by the sulfurous acid fixing baths. The colorized fuchsin-sulfurous reagent stains tissue intensely, including red blood cells, unless the concentration of dye is very low. Tissue stained with basic fuchsin and acidified fuchsin both have a different hue from those with the Feulgen reaction. Although there are some similarities in these stains, as compared with the Feulgen technic, the staining with colorized fuchsin-sulfurous-acid represents a more non-specific staining with a similar pigment, whereas the other two dyes are less specific stains with different pigments.

Several experiments were conducted in which an attempt was made to remove the thymonucleic acid from the nuclei by prolonged hydrolysis, to demonstrate that the chromatin was Feulgen-negative, and then absorb thymonucleic acid upon the chromatin, and finally produce a positive Feulgen reaction. If the protein of the chromatin were sufficiently stable to hydrolysis and if thymonucleic acid or its breakdown products were selectively adsorbed by chromatin as suggested by Stedman and Stedman, such a reaction might be obtained. Accordingly, normal mouse liver, spleen, pancreas and skin each preserved in Zenker-formol, sublimate alcohol, Bouin's, formaldehyde and acetone were sectioned at  $7\mu$  thickness. Tissues were hydrolyzed either forty or sixty minutes at  $60^{\circ}\text{C}.$ ; washed in tap water fifteen minutes; incubated in (a) distilled water or (b) a 1% solution of thymonucleic acid split by desoxyribonuclease or (c) in a 1% solution of thymonucleic acid, each at  $37^{\circ}\text{C}.$  for either one or sixteen hours; washed in tap water fifteen minutes; and stained by the Feulgen technic or hematoxylin and eosin. Control sections were stained by the regular Feulgen technic with only five minutes hydrolysis at  $60^{\circ}\text{C}.$  and with hematoxylin and eosin. Depending upon the fixative employed, the tissues showed varying degrees of loss of Feulgen staining with prolonged hydrolysis. Tissue fixed in acetone lost its ability to show a Feulgen stain after forty minutes and after sixty minutes there was complete or nearly complete loss of staining in tissues fixed in sublimate-alcohol, Bouin's and in formaldehyde. The Zenker-fixed tissue maintained its Feulgen staining property slightly better. All sections were essentially Feulgen-negative after sixty minutes hydrolysis followed by sixteen hours in distilled water. Sections incubated in mixtures of thymonucleic acid or its constituents in some cases showed weak diffuse cytoplasmic staining but no distinct nuclear staining, despite the fact that remnants of the chromatin could still be stained with reduced clarity and intensity by hematoxylin. Thus with the methods employed we were unable to demonstrate adsorption of nucleic acid upon the chromatin.

An additional method was employed to attempt to show that thymonucleic acid is responsible for the Feulgen reaction. If desoxyribonuclease produces a specific breakdown of thymonucleic acid, chromatin treated with this enzyme should be Feulgen-negative. McCarty (1945) observed such a loss of Feulgen staining in liver nuclei isolated by citric acid. He kindly supplied some of his purified preparations No. 17 and 21 (McCarty, 1946) together with suggestions for their use. Our first experiments on paraffin sections

were unsuccessful. There was only a slight diminution in the intensity of the Feulgen stain and many sections came off the glass slides, suggesting some action of the enzyme upon the albumin fixative. In the concentration used, the preparation was said not to be proteolytic (McCarty, 1945, 1946). In the final experiments employing frozen sections better results were obtained. The purified desoxyribonuclease was used in a concentration of 1 mg./ml. in a 1% solution of gelatin in  $M/0.003$   $MgSO_4$ . The gelatin acts as a preservative for the enzyme which is activated by magnesium ions. This enzyme produced a very rapid reduction of the viscosity of solutions of thymonucleic acid. Sections of mouse liver and kidney were incubated at 37°C. for three and sixteen hours. Some of the cells of the tissue fixed in formaldehyde showed a diminution in their Feulgen staining properties whereas most unfixed cells showed complete loss of staining. However, in each instance a few nuclei could be found which still took some stain. Most of the few cells which stained were at the periphery of the tissue where the cellular nucleoproteins could have been altered to reduce their susceptibility to enzymatic action. It is also possible that under some conditions the potential aldehyde groups of the nucleic acid remained in a form in which they could still react to give some Feulgen staining. Unfortunately it was not possible to conduct additional experiments at this time to ascertain more definitely the cause of this slight discrepancy.

Carr suggests that it should be possible to block the reaction of fuchsin-sulfurous-acid with aldehydes by the formation of aldehyde-bisulfite addition compounds in the presence of an excess of sulfur dioxide. However, such sulfur dioxide would also be in equilibrium with the fuchsin-sulfurous compound. Although some of the details of the chemistry of the Feulgen reaction are not clearly understood, it is believed that the sulfur dioxide reacts with and reduces the basic fuchsin to form the colorless leuco-fuchsin or fuchsin-sulfurous-acid reagent. According to Wieland and Schening (1921), the aldehyde groups liberated by hydrolysis react with the colorless fuchsin-sulfurous reagent to form a colored addition compound. Baker (1942) has given structural formulas of the colorless and regenerated fuchsin-sulfurous-acid and attributes the color to the reformation of the quinoid linkage. Caspersson (1932) found that the fuchsin-sulfurous-acid reagent contains an excess of many times as much sulfur dioxide as would be used in the chemical reaction and showed that even a much greater excess of sulfur dioxide would not be expected to significantly inhibit the staining process.

Feulgen (1927) interpreted the fact that the staining reaction was prevented by treatment with ammoniacal silver solution and bromine as evidence that the reaction was thus blocked between the aldehyde groups of thymic acid and fuchsin-sulfurous acid.

#### EVIDENCE SUPPORTING SPECIFICITY OF THE REACTION

In discussing the evidence for the specificity of the Feulgen reaction only some of the more important facts will be mentioned. The basis for the claims of specificity was established by Feulgen and his associates Rossenbeck, Voit and Imhäuser (1924, 1924a, 1924b); for more complete bibliography see Feulgen (1932). It has since been reiterated and augmented by many others including Wermel (1927), Lison (1936), Hillary (1939) and Stowell (1945). The chief claims for specificity are that the properly controlled reaction stains only thymonucleic acid and that the location within the cell of the stain and thymonucleic acid are the same. It is readily demonstrated that the Schiff reaction as modified in the Feulgen technic reacts with thymonucleic acid only after controlled hydrolysis in HCl. Feulgen wrote on paper with a solution of thymonucleic acid and showed that only the impregnated areas were stained. As further evidence of the localization of the nucleic acid and stain, he showed that the thymonucleic acid did not diffuse from the chromatin after hydrolysis.

When the reaction is properly controlled the only parts of cells which stain are areas of chromatin identical with those absorbing ultraviolet light characteristic of nucleic acids (Caspersson, 1939, 1940, 1944 and Caspersson and Santesson, 1942). Claude and Potter (1943) extracted chromatin threads from leukemic blood cells, found they contained 40% thymonucleic acid and stained by the Feulgen technic. After the removal of thymonucleic acid from intact cells by enzymatic action, the Feulgen reaction becomes negative, although the protein framework of the chromatin can still be stained. This has been demonstrated by Mazia and Jaeger (1939), Serra (1943), Brachet (1945), and McCarty (1945), as well as in the experiments reported in this paper. Enzymes which break down ribose nucleic acid, on the other hand, do not effect the Feulgen reaction on chromatin.

#### EVIDENCE AGAINST SPECIFICITY OF THE REACTION

More discussion of the evidence against the specificity of the Feulgen technic will be given because this evidence is more poorly established and generally not accepted. The Feulgen technic

is a histochemical modification of the Schiff test for aldehydes and there are many substances in cells containing aldehydes. It is recognized that materials such as lignin, suberin, cutin, starch, glycogen, and some other polysaccharides may stain with fuchsin-sulfurous-acid reagent (Hillary, 1939). This criticism is answered by pointing out that these substances react in the absence of preliminary hydrolysis which is an essential part of the Feulgen reaction and can hence be detected in unhydrolyzed control sections. Certain cytoplasmic lipids react with fuchsin-sulfurous-acid to give a plasmal reaction (Feulgen and Voit, 1924c; Feulgen and Bersin, 1939) yet they can be differentiated by their location within the cytoplasm and their staining avoided by regularly leaving tissues for 24 hours in 95% ethyl alcohol to remove the lipids. Certain alkaline substances, including purines, stain with the Feulgen reagent (Semmens, 1940), but this reaction does not occur when the hydrogen-ion concentration is regulated properly, as shown by Feulgen and Rossenbeck (1924) and reiterated by Barber and Price (1940). The theoretical possibility that substances other than thymonucleic acid could give a positive reaction may be admitted; however, under properly controlled conditions, it has not been possible to prove that any substance other than nucleic acid of the desoxyribose type is stained.

It has been suggested that the typical reddish-purple pigment which has been produced by the fuchsin-sulfurous-acid reagent and aldehyde groups liberated by hydrolysis might be adsorbed upon structures within the cell which do not contain thymonucleic acid in the living state. That this is improbable is shown by the experiments of Feulgen and the other evidence for the identical location of the Feulgen stain and thymonucleic acid quoted in the preceding section on the support for the specificity of the reaction. Such non-specific adsorption, however, can occur with erroneous technic such as inadequate washing in sulfurous acid or the use of old fuchsin-sulfurous-acid reagent or sulfurous acid bleaching baths in which the concentration of sulfur dioxide is low. Our own experiments have confirmed this observation of other workers.

The presence of malarial pigment can interfere with the Feulgen technic; however, Deane (1945) has described precautions to obtain a positive Feulgen reaction on malarial parasites.

Fischer (1942) has pointed out that since thymotetranucleotides, in contrast to polynucleotides, are soluble in aqueous and acidic solutions, they would not give a positive Feulgen reaction. However, the amount of such easily soluble nucleotides within tissues are not

great and their significance and functional relation to desoxyribopolynucleotides are not well established. Therefore, the fact that such nucleotides do not stain contributes to, instead of detracts from, the specificity of the Feulgen reaction for thymonucleic acid.

It is said that since some of the details of the chemistry of the Feulgen reaction are not understood, therefore its specificity can only be presumed. To entirely accept such a statement one must completely ignore the considerable amount of evidence for the specificity mentioned above. Some details may not be understood better until our knowledge of the molecular reactions of nucleoproteins and dyes is more complete. In the meantime, realizing the limitations of our present knowledge and that our opinions may change with further evidence, it seems reasonable to accept as quite probable the relative specificity of the Feulgen technic.

Aside from the question of its specificity, if modified staining methods could give entirely comparable results with less effort, their use would be justified. Experiments in this and a previous paper (Stowell, 1945) describe certain superficial similarities as well as important differences in the reaction of fuchsin dyes with the tissues. Naturally, when making such comparisons, one must recognize the difficulty of obtaining different stains in equivalent concentrations either in solutions or in tissues. As contrasted with the regular Feulgen technic, the fuchsin-sulfurous-acid reagent which has been colorized by the addition of aldehyde (Choudhuri, 1943) has similar hue and spectrophotometric absorption curves. Even in moderate concentrations, it stains rapidly and intensely structures such as cytoplasm, nucleoli, keratin and red blood cells which are not stained by the properly controlled Feulgen technic. A preliminary short acid hydrolysis is unessential and, after prolonged hydrolysis has made chromatin Feulgen-negative, the residual protein can still be stained with the colorized fuchsin-sulfurous-acid reagent. The stained tissue fades more rapidly on exposure to a carbon arc light. Similar comparisons between the regular Feulgen technic and acidified basic fuchsin (Carr, 1945) show that the latter has a slightly different hue. Only in the weakest concentrations of acidified basic fuchsin can one obtain similar staining of chromatin, nucleoli, and cytoplasm. Preliminary acid hydrolysis is unessential and the acidified fuchsin tends to stain more structures more rapidly, including cytoplasm, and is more readily washed out of sections by water, alcohol and sulfurous acid solutions. These experiments do not support the view that either of these suggested technics gives equivalent results to the Feulgen reaction.

Until our understanding of the nature of the Feulgen reaction is more complete we may continue to evaluate it critically; however, it cannot be overemphasized that before discussing its specificity writers should thoroughly evaluate the available information in the literature. The preponderance of evidence indicates that with the proper precautions the Feulgen technic for thymonucleic acid is one of the most specific histochemical reactions.

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## ADDENDUM

Of the papers published on the Feulgen reaction since this manuscript was prepared, three are of especial interest. Brachet (1946) answers the criticisms of Stedman and Stedman (1943a, 1943b, 1944) and Carr (1945) by presenting experimental evidence that following the treatment of cells with thymonucleodepolymerase the Feulgen

reaction is negative, that acid hydrolysis does not destroy the cytoplasm so that only chromatin stains and that acid hydrolysis of chromatin does not liberate diffusible aldehyde groups. Dobson (1946), who also disagrees with Carr's claims, similarly produced Feulgen negative chromosomes with an intact protein framework by the action of nuclease. He showed that the hydrolysis of fixed tissues did not produce appreciable change in their dry weight as one would expect from the dissolution of large amounts of cytoplasm. Both Dobson and Brachet offer suggestions why an excess of  $\text{SO}_2$  should not block the Feulgen reaction as Carr claimed.

Stacey, Deriaz, Teece and Wiggins (1946) suggest that a positive Feulgen reaction is produced by the reaction of fuchsin-sulfurous-acid with  $\omega$ -hydroxyl levulinic aldehyde, as well as two substances from which it may be derived, l-arabinal and l-deoxyribose. Contrary to histochemical observations, such a small molecule of aldehyde would be expected to diffuse freely from the cell and to give a poorly localized Feulgen reaction. Brachet's observation (1946) that the supernatant of the hydrolyzed thymonucleoprotein of cells gave a negative Dische reaction is also inconsistent. Before attempting to interpret this macrochemical work of Stacey and his coworkers in terms of the Feulgen technic on tissues, additional observations would be desirable.

BRACHET, J. 1946. La spécificité de la réaction de Feulgen pour la détection de l'acid thymonucléique. *Experientia*, 2, 142-3.

DOBSON, E. O. 1946. Some evidence for the specificity of the Feulgen reaction. *Stain Techn.*, 21, 103-6.

STACEY, M., DERIAZ, R. E., TEECE, E. G., and WIGGINS, L. F. 1946. Chemistry of the Feulgen and Dische nucleal reactions. *Nature*, 157, 740-1.

## A NEW TECHNIC: TANNIN-IRON II

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This technic is hereby called "tannin-iron II", in order to distinguish it from the first technic with tannic acid and iron, which we now call "tannin-iron I" (Stain Techn. 19, 1944). The technic of tannin-iron II consists of successive mordanting in tannic acid and in iron alum, repeated  $n$  times, with washing in water between each bath.

The baths of tannin and iron alum are the same as those for tannin-iron I:

1. "Acetic tannin"

Tannic acid, 6 grams  
Glacial acetic acid, 10 ml.  
Distilled water, 30 ml.

2. 4% aqueous ferric ammonium sulfate

The reaction takes place progressively through the passages resulting in total blackening of the section when the reaction is complete.

After the complete reaction or after even a partial reaction (i. e. repeated only 2, 3, or 4 times), one can differentiate with hydrochloric alcohol, tannic acid, or "picric tannin" (i. e. tannic acid, 25 g.; picric acid, 0.1 g.; distilled water, 100 ml.). The total Reaction II is not  $n$  times Reaction I; it is a totally different reaction. It is positive with any fixative: with alcoholic tannin; after the action of heat; after the action of ammonia, etc.

All this is the opposite of Reaction I. In Reaction I chemical processes dominate; in Reaction II physical processes dominate.

In Reaction II there is a complex interference between the processes of mordanting and of differentiating. Reaction II either complete or partially complete can give rise to several new methods: "Safranin-tannin-iron-II": "Azocarmine-tannin-iron-II": "Giemsa-tannin-iron-II", etc.

Tannin-iron II, either complete or partially complete, gives entirely different results from Reaction I. But, like Reaction I, it colors only proteins; it never colors lipids or even lipoproteins. Reaction II gives new pictures of collagen; it never colors elastin. It permits the making of a study on the architecture of the cytoplasm. It

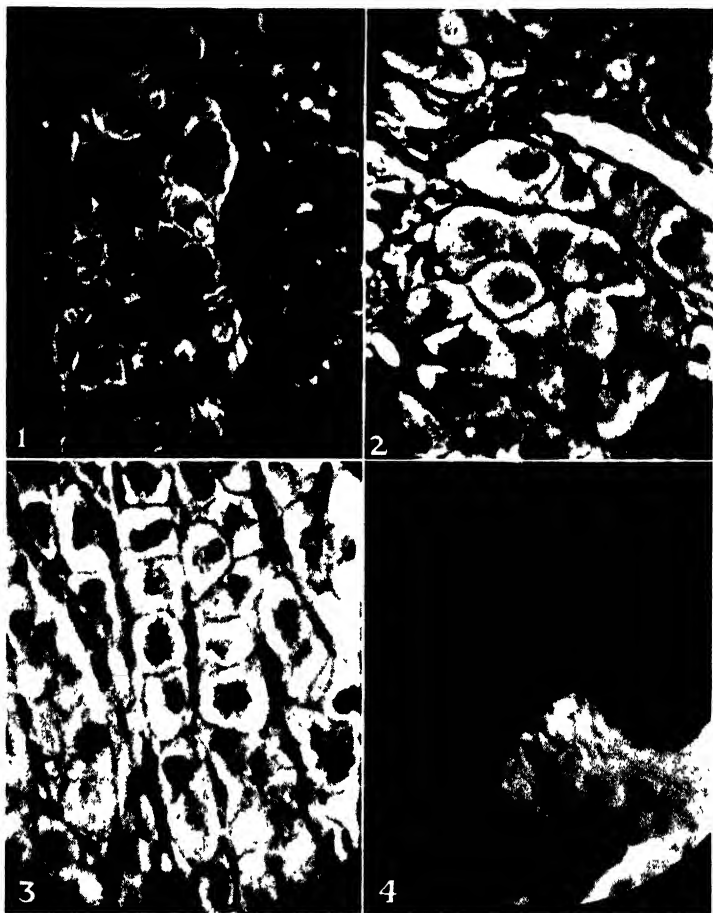


FIG. 1. Pancreas, Bouin, Tannin-iron-II; total reaction; differentiation at the beginning. Several pancreatic cells and some of the cells of the ganglia are already at total reaction (black). Others are half differentiated. Several, in the ganglion, are already differentiated with the para-Golgi apparatus.

FIG. 2. Ovary, rabbit; Bouin, Tannin-iron-II; total reaction with moderate differentiation. The cells are almost completely differentiated. Only the Zone of Golgi and some nuclei are still black.

FIG. 3. Ovary, rabbit; Bouin, Tannin-iron-II; total reaction with very strong differentiation. In all cells the para-Golgi apparatus is black and the zone of Golgi a clear gray.

FIG. 4. Human skin. Bouin, Tannin-iron-II; total reaction, moderate differentiation. The conjunctive papilla is already differentiated, the epithelium almost differentiated with the exception of the basal cells, which are still black.

gives pictures of para-Golgi analogous to those one obtains with Reaction I, but obtained by regressive staining.

Reaction II has already given remarkable and new results in animal cytology, normal and pathological, and in plant cytology, and in the cytology of tumors. The results are not yet published. It possesses also great interest on the physicochemical side and on the theories of mordanting.

We wish to state that one can blacken a sheet of glass or of metal with the completed Reaction II. In practice, Reaction II presents a great adaptability, for one can employ it in complete or partial reaction, with or without differentiation, unstained or with stains. The reaction does not depend on the concentration or the duration of the baths. It depends only on the number of passages. In order to bring this about, it is necessary to pass the sections through the baths. The number of passages required for the complete reaction varies with the fixative, the tissue, etc. In practice it is accomplished when the section is black and the slide begins to turn blue outside the section.



## A ONE-SOLUTION TANNIC-ACID-IRON STAIN FOR PLANT TISSUE SECTIONS

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**ABSTRACT.**—After completing the bulletin on "Actinomycetes in various parts of the potato and other plants" (Lutman, 1945) the author found the beautiful plates in the atlas to Olivier's monograph (1881) on root structure in which the same intercellular inclusions were shown. Olivier stated that he had stained his sections in "tannate of iron". Attempts were made by the author to prepare and use such a combination but they were unsuccessful owing to the precipitate that was formed.

The formula used by the U. S. government for ink for official use was tried. This combination is composed of tannic and gallic acids with ferrous sulphate and is acidified with hydrochloric acid. When used double strength, as suggested for special blackness and permanence, the stain was very successful on sections of potato roots and tubers. It stained the Actinomyces hyphae very differentially and was decolorized from all other cell organs. Any other stains used dyed also the pectins and the Actinomyces secretions (melanins) but with this iron tannate combination in one solution, the finest hyphae could be seen and photographed. Since hydrochloric acid was used in this stain, such Actinomyces inclusions must be very tannophylic; much more so than any animal intercellular inclusions so far described.

Tannic acid dissolved in dilute acetic acid and followed by a dilute iron chloride solution has many advantages over other stains in differentiating the filamentous, intercellular inclusions of plant tissues identified by the author (1945) as the hyphae of Actinomycetes. The Derrien and Turchini (1924) modification of the Salazar (1920) technic gave excellent results on a variety of plant materials.

After the bulletin (1945) was in type, the author found the beautifully illustrated atlas to Olivier's monograph (1881) on the structure of the outer layers of the roots of plants. Olivier's photomicrographs, reproduced by a gelatin process, showed the Actinomyces hyphae stained so brilliantly that his interest was aroused to look up his technic. To his surprise, Olivier stated that the sections, which had been cut in part by hand and in part on a microtome, had been

stained with "iron tannate" to bring out the walls more clearly, but no details on methods were given as they were apparently too well known.

A number of trials by the author to make "iron tannate" in the laboratory were unsuccessful. Much of the combination precipitated out although the solution was acid. The precipitation of the iron tannate by the addition of ammonia to make the solution alkaline, followed by a filtering out and a redissolving of the black precipitate was even more unsuccessful. The precipitate could not be dissolved in an acid weak enough to use on plant sections.

These failures resulted in a search through the older literature to find any iron and tannic acid combinations in use in the 1860-1885 period. The introduction of the anilin dyes with their brilliance and variety of color and reaction about the latter part of this period, undoubtedly checked the development and use of the iron-tannic acid stain. Since the latter is such a valuable complement to the anilin dyes, a short review of the early use and variations of this combination may interest stain technologists. While many of these combinations were microchemical tests for tannin, others included the use of tannin or iron chloride solutions as fixing or hardening agents with any staining as a secondary consideration.

Poulsen (1883), quoting Hohnel, states that according to Pliny, the first chemical reaction known to the ancients was that of tannin and iron salts: "the characteristic reaction of a decoction of galls with iron was used by the ancients to detect the adulteration of verdigris with sulfate of iron." The first suggestion for the use of iron chloride as a test for tannin in plant tissues was made by Link in 1807.

Poulsen used iron salts as a microchemical test for tannin in plant cells, the acetate or sulfate being recommended rather than the chloride in the test solution because the tannate of iron which is formed is more soluble in an excess of the iron chloride than are solutions of iron acetate or iron sulfate in their iron salts.

Either tannic acid or iron chloride was a constituent of some of the early fixing solutions according to Whitman (1885). The hint that led microscopists to use tannin was undoubtedly the tanning of hides by a tan bark, a process in which tannic acid (and gallic) coagulated and hardened the proteins. Carnoy had, according to Whitman, used tannic acid in a 0.5% solution for fixation in 1886.

But, even earlier, Vulpian in 1856 on nerve tissues and Fol (p. 102, 1884) had recognized iron chloride as an excellent fixing agent; the latter author recommending it for ciliated epithelium and pseudo-

Pods. The pharmaceutical tincture of iron chloride was diluted with 5 to 10 times its volume of 70% alcohol. The fine precipitate which formed, redissolved when a few drops of HCl was added and the mixture vigorously shaken. Penetration was poor so only small pieces of tissue could be properly fixed.

Whitman recommends "the best method of staining such objects is to add a few drops of gallic acid (one per cent solution) to the alcohol. After 24 hours, the acidulated alcohol is poured off and the pure alcohol is added. Thus treated, the protoplasm will take a light brown color, the nuclei a much deeper brown. Carmine stains too deeply and diffusely, and cannot be successfully removed."

Robin (1871) recommended iron chloride (the tincture diluted to the color of port wine) for certain tissues as a coagulant and cites Ordoñez (1865) who preserved and stained skin or glands in a mixture of white glycerine, 25 grams; distilled water, 10 grams; and tannin crystals, 20 milligrams. The specimens were stained a maroon color in part, or were at least darkened so that the study of cell details was aided.

Beale (1865) states that "although tannin does not colour animal membrane, it alters its character to such an extent as to enable us to see many peculiar points of structure or arrangement not visible before, or it produces a chemical change in the substance from which we can gain important information. Solutions of magenta and tannin have been much used in investigations on blood corpuscles." The tannin solution was made by dissolving 3 grains of tannin in an ounce of water.

Nageli (1867) recommended a very weak solution of any iron salt for plant cells containing tannin, the only precaution being an immediate observation of the section since the tannin is soluble in the aqueous solutions.

Two other uses of tannin or iron salts should be pointed out but need not be discussed in detail. Tannic acid solutions as a coagulant and mordant for the very delicate and aqueous proteins of bacterial flagella were developed about this period and Heidenhain used iron alum as a mordant for hematoxylin.

While the essentials for the use of tannin and iron salts may be all found in older technics, some of which used one of these chemicals as a fixing agent, it remained for Salazar (1920, 1923, 1944) to combine them into a mordant and stain that is very specific for the intercellular inclusions of animal tissues. His theory is that the tannin, dissolved in acetic acid (one part acid to two of water), was a compound of definite composition (*tannin acetique*). It acts as a

mordant on the "tannophylic" elements as a coagulant and precipitant. The subsequent staining of this coagulated core by iron salts (an iron alum solution) was a chemical reaction and not simply a physical deposition of iron tannate. Hydrochloric acid, even in weak solutions, bleached all color from the sections but they could be restained by repeating his original procedure.

The colors obtained varied from a gray to a pure black but with various shades of blue from a pale to a deep blue. These shades of color intensity were measures of the tannophylic properties of the cell components. The tissues were all from animal sources. The author (1945) would state here that the colors obtained on plant tissues for the intercellular inclusions which he believes to be the hyphae of Actinomycetes were a deep steel-blue or a pure black while the cytoplasm was a light brown and the chromatin of the nuclei a dark brown.

But Olivier (1881) had stated that he used iron tannate on his plant sections. The thought then occurred to the author that perhaps the old type of writing ink in use before anilin dyes were introduced into ink manufacture might serve the purpose since such solutions must have been known to Olivier. The older type of inks was made from nut galls and iron salts. It was almost colorless and the black insoluble salt that was formed in a few days of exposure to air and light was a mixture of iron tannate and gallate according to the older chemistries.

The formula for the writing ink supplied by the Bureau of Printing and Engraving to all U. S. departments and the procedure for making it, follows:

Tannic acid (tannin) . . . . .	23.4 grams
Gallic acid. . . . .	7.6 "
Ferrous sulphate. . . . .	30.0 "
HCl (dilute, U.S.P.) . . . . .	25.0 "
Phenol . . . . .	1.0 gram
Water, to make up to. . . . .	1,000 ml.

The formula given above is that for special permanence and blackness. The first four constituents: tannic and gallic acids, ferrous sulfate, and hydrochloric acid, are double the amount contained in the "standard" formula. In the government recipe, 3.5 grams of "soluble blue", known also as "cotton blue" or "Schulte's 539", is added to provide a light blue color so that the writing may be at once visible. In the stain, of course, no anilin dye was added. To make the stain:



FIG. 1. Potato tissues stained with iron tannate

- A. Longitudinal section of potato root tip. The stained hyphae extend up and down the root; the only other organs stained are the nucleoli.
- B. Section of cork regenerating on an old potato tuber. The delicate filaments which invade the new cambium are brought out in full detail.  $\times 400$ .

- A. Dissolve tannic and gallic acids in 400 ml. of water, preferably distilled or rain water.
- B. Dissolve ferrous sulfate in 200 ml. warm water to which HCl has been added.
- C. Dissolve the phenol in 200 ml. water. (For ink, add the blue dye to this solution.)

Mix the three solutions. Cool to room temperature. Make up to 1,000 ml. by using the wash water of the three containers. The liquid will be almost clear and colorless with no precipitate but will color slightly after standing a few days. Place, without shaking, in sealed bottles if it is to be used some time later.

Potato roots and tubers, fixed in Allen-Bouin's solution, and cut in paraffin were stained by this iron tannate solution. The sections were transferred from water to the stain; 1 to 2 hours seemed to give the best results. No special precautions were necessary on the time since no overstaining seemed to be possible even on sections left 24 to 48 hours. In fact, the sections did not seem to be colored at all as they were removed from the stain and much of the stain was washed out in the absolute alcohol which must be applied as rapidly as possible; the only precaution necessary for success. One should clear in clove oil and mount in Canada balsam as usual.

The special advantages of this stain over any other tried on plant material is that the *Actinomyces* hyphae are left stained while the material in which they are imbedded and all other cell organs are decolorized. Many *Actinomycetes* secrete a brown pigment which diffuses into the adjacent walls and cytoplasm. These brown pigments (melanins) absorb stain, not as well as the hyphae from which they originate, but enough to make decolorization and photography difficult. Some absorption of the stain by the pigment occurred even in the combination of tannic acid and iron chloride previously used, although less so than in the modified Gram stain (Hutchins and Lutman, 1941) by which the hyphae were first seen, unless the decolorization is carried to exactly the correct stage. With iron tannate used as a one-stain solution, the finest branches of the hyphae may be traced as the accompanying figures show, especially B, in which the hyphae are invading the young cork cambium developed on a mature potato tuber after injury by cutting. The nuclei of the root tip cells of A are not stained; the shading is due to the reflection of the light from the walls of the nuclei and cells.

The iron tannate stain is brilliant and permanent. Unless all clove oil is removed by a subsequent immersion of the sections in

xylene, crystal violet will fade from the finer hyphae; iron tannate, on the contrary, becomes darker as time passes.

The combination of iron and tannic acid, used as two solutions, has been found to be very useful in differentiating intercellular materials in both animals and plants. In animal tissues, the tannic acid seems to have an affinity for and to be held by the intercellular collogens (gelatin-producing substances). In plant tissues, the stain reacts with the pectins and the included filamentous hyphae of the Actinomycetes. The single solution, iron tannate stain and mordant, used on plant tissue, seems to be held by these hyphae only. For this reason it should be very useful in tracing the intrusion of the hyphae between the walls of cells which have just divided.

The successful use in a single solution of tannic and gallic acids with iron salts and hydrochloric acid indicates that in plants, at least, some of the intercellular elements may remain stained. Salazar (1920) had found that even a dilute solution of hydrochloric acid would remove the stain. The intercellular filaments which remain stained in this one solution must, therefore, be much more "tannophilic", to use the Salazar term, than anything included between the animal cells which he stained. The author doubts the existence of the compound, "acetic tannin", but is certain that the addition of acetic acid to the tannin solution aids in the mordanting of some of the cell components which would otherwise remain unstained when the sections are transferred to the iron salt solution. The one solution, mordant and stain, is recommended, however, for the differentiation of the Actinomycetes hyphae between plant cells.

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## BLOCK-SURFACE STAINING<sup>1</sup>

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**ABSTRACT.**—A method is described by which the tissue exposed on sectioning a specimen embedded in paraffin can be visualized *in situ*. The fixed specimen is impregnated with lead acetate, dehydrated in dioxane, infiltrated with paraffin and embedded. Tissues exposed on sectioning are developed by applying to the cut surface of the block a solution of potassium sulphide in water. Concentrations of the reagents used and the time intervals for the procedure are dependent upon the size of the specimen and upon the degree of contrast required. The method is described as it was applied to the study of a small human fetus in cross section. Representative photographs are included to show the results obtained.

### PROCEDURE

The following departure from routine methods was used in studying the anatomy of a 30 mm. human fetus in cross section. The specimen had previously been fixed in 10% formalin and stored for about eight months in 75% alcohol.

The fetus was washed in running water for 6 hours and then left for 24 hours in a 50% saturated solution of lead acetate in water. Dehydration was carried out in dioxane (2 changes in 24 hours). The specimen was then infiltrated in a solution of dioxane and paraffin for 3 hours, pure paraffin for 4 hours, and embedded.

The block was mounted vertically in a sliding-knife microtome and sectioning was begun in the usual manner. Sections passing through the embedded specimen were visualized *in situ* by applying to the cut surface of the block a drop of a 40% saturated solution of potassium sulphide in water. (The addition of dioxane to this solution as originally was recommended<sup>2</sup> is not required.) Observations could then be made of the block surface (Fig. 1).

Serial levels were prepared by sectioning and treating the block surface at each new plane in the manner prescribed (Fig. 2 and 3).

By this method the tissue on the exposed surface of the block

<sup>1</sup>For helpful suggestions the writers are indebted to Dr. Robert W. Ramsey of the Department of Physiology, Medical College of Virginia, and to Dr. K. L. Kaufman and Dr. Milton E. Neuroth of the Medical College of Virginia, School of Pharmacy.

<sup>2</sup>Hegre, Erling S., and Brashear, Alton D. 1946. On visualizing the cut surface of tissue embedded in a paraffin block. *Anat. Rec.*, **94**, 94. (Abstract.)

**PLATE 1. EXPLANATION OF FIGURES**

Photographs of sections through a 30 mm. human fetus embedded in paraffin as seen on the exposed surface of the block after precipitation of lead sulphide.

1. Block surface. Contact print of a Kodak contrast process pan negative. Magnification 6 times.

2. Block surface. Negative enlargement of a single frame from a 16 mm. reversible film. Total magnification about 4.5 times.

3. Block surface. Negative enlargement of a single frame from a 16 mm. film taken at an initial enlargement greater than that shown in figure 2. Total magnification about 7.5 times.



is clearly outlined by a black precipitate of lead sulphide. It therefore stands in sharp contrast to the unstained embedding medium. The precipitate is found in the tissues to a depth of from 5 to 15 $\mu$ , depending upon the duration of the potassium sulphide exposure. Other factors controlling the depth of penetration have not yet been determined but among these may be the density of the paraffin and the amount of lead acetate in the tissue.

The addition of various substances to the embedding medium to produce a whiter background is contemplated. Efforts to obtain a maximum contrast are prompted by the thought of producing a photographic film record of a complete series of undistorted levels through an embryo.

## LABORATORY HINTS FROM THE LITERATURE

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A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS  
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

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### MICROSCOPE AND OTHER APPARATUS

BENNETT, A. H., JUPNIK, H., OSTERBERG, H., and RICHARDS, O. W.  
**Phase microscopy.** *Trans. Amer. Micr. Soc.*, **65**, 99-131. 1946.

This well illustrated paper describes the apparatus and methods for this interesting type of microscopy and some of the observations made with it.—*V. Kavanagh*.

HINSCHKE, GEORG. **Technik und Anwendungsgebiete der Intralumination-methode zur Beobachtung innerer Organe am lebenden Tier.** *Zts. Wiss. Mikr.*, **59**, 131-41. 1943.

The position, form, and function of internal organs may be observed in the living animal by means of x-rays, endoscopy, contrast media and fluorescence processes as well as with surgical methods. The author believes the non-operative technics pose advantages by preserving normal relations and conditions, their only disadvantage being the limited application, loss of color and plasticity, and other disturbances. The author recommends his method of "intralumination". He suggests the use of small pencil light bulbs mounted on suitably flexible leads. These can be used directly, or may be placed in glass tubes for insertion in the mouth, esophagus, stomach, etc. of amphibians and other animals. Observations are made in a darkened room. Among the advantages of this method are prolonged and continuous observation without injury to the animal, a better demonstration of dimensions, and observations of the lungs not demonstrable by any other method. Lastly, this method may also be used to visualize and circumscribe the operative field.—*J. M. Thuringer*.

### MICROTECHNIC IN GENERAL

GREEN, HENRY. **Two-dimensional form of flocculation.** *Ind. Eng. Chem.*, **38**, 679-82. 1946.

In making particle size measurements with the microscope, the investigator is often confronted with the problem of deciding just what constitutes the ultimate particle. The technic for studying the structure of a pigment vehicle suspension consists in placing a small drop (1 or 2 mg.) of the material on a slide and covering it with some of the vehicle from which the suspension was made. A cover glass is placed on top and pressed down rather firmly. Microscopic examination of the edge of the flattened drop shows the state in which the pigment exists. Particle aggregation is indicated if pressure on the cover glass causes the material to deflocculate, wrinkle, or tear.—*R. T. Whittenberger*.

O'BRIEN, H. C., JR., and McBAIN, J. W. **Thin undistorted sections for electron microscopy.** *J. Amer. Chem. Soc.*, **68**, 1139. 1946.

Sections  $0.1\ \mu$  thick are cut by means of a high speed ultramicrotome. An electron micrograph of a soap section is presented to demonstrate that the ultramicrotome can yield thin sections of a soft plastic body without smearing or distortion. The sections are caught directly upon 200-mesh wire gauze without coming in contact with any surface, and are then examined.—*R. T. Whittenberger*.

WELCH, L. M., ROSEVEARE, W. E., and MARK, H. Fibrillar structure of rayon fibers. *Ind. Eng. Chem.*, 38, 580-2. 1946.

The specimen is mounted on a slide and covered with a cover glass. A small amount of 70% nitric acid is placed at the edge of the cover glass in contact with the specimen, which swells as the acid penetrates. Application of slight pressure on the cover glass causes the swollen fiber to disintegrate into fibrils whose arrangement with respect to the original fiber axis is easily seen.—*R. T. Whittenberger.*

### ANIMAL MICROTECHNIC

HIRSCHLER, J. Grundsätzliches über Osmiumfixierung und Osmiumfärbung. *Zts. Wiss. Mikr.*, 59, 113-39. 1943.

According to Hirschler, it was v. Berger's attempt to correlate the morphology of the Golgi-apparatus with cell physiology that inspired E. Sjövall to investigate "the conditions for the successful and rational use of the osmic acid method". Hirschler regrets that Sjövall's original work (*Anat. Hefte*, 30. 1906) is but rarely mentioned in most recent investigations.

In a series of experiments, Sjövall exposed the Golgi-apparatus to the same osmic acid solution under various, strictly controlled conditions until he repeatedly obtained uniform results. He attributed the difference in the appearance of the Golgi-apparatus in the peripheral and inner zones of the tissue to the diminishing concentration of the precipitating osmium tetroxide with its low diffusibility. He explained the Golgi-cycle of v. Bergen on the basis of this "zone effect". The presence of dissimilar tissues adjacent to cells in which the Golgi-apparatus was impregnated (e.g., ganglion cells associated with myelinated nerve fibers on the periphery of a ganglion) showed beautiful impregnation to a point where the cells left the proximity of the nerve bundles. From there it became less precise as the distance to these nerve bundles increased. This "neighbor effect" demonstrates the influence produced by other osmic acid reducing substances, such as myelin sheaths and fat, on the success of the osmication of the Golgi-apparatus in spinal ganglion cells.

Sjövall emphasized that the variations in the morphological picture bear a direct relationship to the degree of concentration in which the osmic acid solution reaches the cell. He considered the Golgi-network to possess the character of a myelogenic substance: "it has the property to hydrolyze which renders it capable of taking up osmium tetroxide which is subsequently reduced". (It might be explained here that the mention of hydrolysis refers to processes of submicroscopic order, which would scarcely influence the microscopic picture unless hydrolyzation had been carried to excess).

Sjövall was the first investigator to divide the combined fixing and osmication of Kopsch into two separate and distinct procedures, a principle which proved exceptionally fruitful and permitted various later modifications extending the Kopsch method to general histological and cytological investigations. Hirschler remarks that: "it is but slightly more complicated than the Kopsch method, requiring the use of a refrigerator and neutral formalin as a fixing agent". Many investigators failed to obtain the clear pictures by the Kopsch method which, according to Hirschler, is evidence of their failure to make use of the "fixation series" as demonstrated by Sjövall. The latter showed rightly that the fixation of the object will influence the appearance and preservation of the Golgi-apparatus and other lipid cell constituents. Postosmication can influence results only to a slight degree or not at all. A single fixation may produce, in serial sections, results which may be due to "zone effects", and to differentiate these a series of fixations must be undertaken.

Sjövall sought a method to eliminate or limit the complicating "zone effects" and to bring about a more uniform blackening of the sections. This search after a new basic technic was intimately tied up with his own experiments, for he stated: "Our search must find a new substance which has only slight coagulating action on albumin and simultaneously preserves the morphology of the cells." These requisites were fulfilled ideally by the osmic acid technic. The search for the ideal fixative led to formalin which he recommended for spinal ganglia of chicks.

In addition to the two principles set forth by Sjövall, his work contains a whole

series of valuable hints which constitute "musts" to all workers in this field.—*J. M. Thuringer.*

**ILLIE, R. D.** Studies on selective staining of collagen with acid anilin dyes. *J. Techn. Methods*, 25, 1-47. 1945.

Various acid azo, triphenylmethane and diphenylnaphthyl methane dyes were tested as selective collagen stains. Naphthol blue-black (C.I. No. 246), fast green FCF, acid fuchsin (C.I. No. 692), methyl blue (C.I. No. 706), anilin blue (C.I. No. 707), wool green S (C.I. No. 737), and violamine R (C.I. No. 758) gave best results as fiber stains in Van Gieson, mixed Masson-Van Gieson, and Masson-Mallory procedures; nitrazine, brilliant croceine, Biebrich scarlet, brilliant purpurin R, fast green FCF, acid fuchsin, wool green S and violamine R excelled as plasma stains in the two latter of the above procedures.

In general picric acid variants surpassed phosphomolybdic and phosphotungstic methods for connective tissue staining. Acidification of picrofuchsin mixtures, with increased content of acid fuchsin, improved both their completeness and selectivity. Picric acid could be used as a mordant between the plasma and collagen stains.

Intermediate mordants could be omitted and differential connective tissue staining still be obtained with methyl blue in acetic acid. HCl was substituted for acetic with superior results.

Selectivity of these stains was best at pH levels below 2.0. Picric acid, phosphotungstic and phosphomolybdic acid methods all appeared to depend on this principle, rather than on any mordant effect.—*C. Randall.*

**THOMAS, J. ANDRÉ.** Technique de coloration des fibrilles collagènes du tissu osseux par l'hématoxyline phosphomolybdique au dioxane. Coloration des gaines limitantes des ostéoplastes. *Bull. d'Histol. Appl.*, 20, 19-24. 1943.

Although the methods of studying connective tissue can usually be adapted to young osseous material, it is much more difficult to handle the fine collagenous fibers of adult bone in this way. The authors have developed a modification of their procedure using phosphomolybdic hematoxylin in dioxane which gives satisfactory results with adult bone. They used thick sections of compact adult bone embedded in celloidin and fixed in formalin.

Their method is as follows: Wash in running water about 1 hr. Reduce in a mixture of acetic acid, 1 ml., 70% alcohol, 99 ml., and pyrogallic acid, 1 g (mix the pyrogallic acid with the acetic alcohol just before using). Wash in distilled water, 15 min. Stain in phosphomolybdic hematoxylin dissolved in dioxane, 15 min. Wash rapidly in distilled water. Differentiate, 10 min. to 1 hr., in a mixture of acetic acid, 1 ml., HCl, 0.5 to 1 ml., and 96% alcohol saturated with picric acid, to make 100 ml. (If the differentiation is too long, the acetic acid and HCl may be added to 100 ml. of a mixture of dioxane and 96% alcohol saturated with picric acid, or instead a mixture of 1 ml. acetic acid and 99 ml. dioxane saturated with picric acid may be used.) Wash in 96% alcohol. Dehydrate, dissolve the celloidin and differentiate in two changes of dioxane for 2 to 5 min. each. Clear in xylene (two changes), 5 to 10 min. each, and in oil of origanum for 5 to 10 min. Mount in balsam.

The collagenous fibers of the bony substance are violet red with the background more or less yellow.

This method with a few modifications may be used to stain the limiting sheaths of osteoplasts. The author says that treatment with the reducing agent is not necessary, that the staining must be very short, around 1 min., and that the differentiation must be severe, using a mixture of acetic acid, 1 ml., and dioxane saturated with picric acid, 99 ml.—*Jean E. Conn.*

## PLANT MICROTECHNIC

**MOORE, RAYMOND J.** Investigations on rubber-bearing plants IV. Cytogenetic studies in *Asclepias* (Tourn.) L. *Canad. J. Res.*, 24, 66-73. 1946.

The somatic chromosome number was determined for *Asclepias syriaca*, *A. incarnata*, *A. incarnata* var. *pulchra*, *A. incarnata* forma *albiflora*, *A. Sullivantii*, *A. tuberosa*, *A. curassavica*, and *A. speciosa* and was found to be 22. Chromo-

some counts were made from fresh smears of growing leaves and root tips as follows: fix in Carnoy's fluid (3 parts chloroform, 2 parts 95% ethanol, 1 part glacial acetic acid); stain in 45% aceto-orcin; heat; flatten on slide. Root tips of *A. curassavica* were fixed in Allen's Bouin fluid, embedded, sectioned, and stained with gentian violet.—*H. P. Riley.*

### MICROORGANISMS

**BUCHERER, HERBERT.** Experimentelle Untersuchungen über die Fluoreszenzfärbung toter und lebender Bakterien nach Strugger. *Zentbl. Bakt., II Abt.*, 106, 81–8. 1943.

Strugger (1941) described a method which he thought distinguished between dead and living bacteria by means of their differential absorption of acridine orange from a 1:10,000 concentration of dye in 0.75% salt solution. The living cells were thought to absorb the dye slowly and to show a green fluorescence while the dead cells took up the dye in large amounts and gave a copper-red fluorescence.

Bucherer performed similar experiments with four species of *Sarcina* and a *Pedococcus* from beer and raises the following objections:

1. Ordinary bacteria are not, as Strugger believed, dead ten minutes after they are smeared on a glass slide, but most of them are alive.

2. Unfixed bacterial smears give the red color with acridine orange, observed by Strugger, not because the bacteria are dead, but because the large amount of dye in solution permits the bacteria to accumulate enough to give the red color. By reducing the amount of dye in proportion to the number of bacteria in smears, either by decreasing the size of the drop or by increasing the number of bacteria, smears were obtained which showed only the green color. Others showed some red and some green.

3. Similar effects were obtained with unsmeared preparations.

4. Bacteria showing the red fluorescence were grown on agar plates and showed no reduction in vitality.

5. Dead bacteria and living ones showed identical reactions to acridine orange.—*V. Kavanagh.*

**PETRIK, F. G.** Atypical acid-fast microorganisms. II. Desoxyribonucleic acid content. *J. Bact.*, 51, 539–45. 1946.

Several species and strains of *Mycobacterium* were analyzed for desoxyribonucleic acid. Possibly the avirulent strains contained smaller quantities than did the virulent.—*V. Kavanagh.*

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# STAIN TECHNOLOGY

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## A RAPID STAINING METHOD FOR THE MICROSCOPICAL EXAMINATION OF MILK.—A PRELIMINARY NOTE

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In the routine examination of milk with the Breed microscopical method, speed in the staining of the smear, coupled with a clear background are important factors if many samples are to be examined.

The standard method requires about seven minutes, more or less. The bacteria and other cells are stained dark blue against a light blue background. However, when sour milk is used, the heat required in the drying of the smear tends to curdle the milk on the slide, with the result that thick dark streaks are often present in the background. These thickened areas often conceal bacteria and other cells which may be present.

A method has been developed whereby the milk smear can be defatted, fixed and stained in one operation in from five to fifteen seconds and in which the background can be completely decolorized.

The method is based upon the rapid staining formula developed by Erb<sup>1</sup> and the principle of background decolorization of Mallman and Churchill.<sup>2</sup> The formula is as follows:

Ether	100 ml.
Methyl alcohol, absolute	100 ml.
Methylene blue	1 g.
Hydrochloric acid	0.7 ml.

The preparation of the formula is important. The ether and the methyl alcohol should first be mixed together before the methylene blue is added. As soon as the dye is in solution the hydrochloric acid can be included. Variations in this order of procedure may result in failure.

<sup>1</sup>Erb, N. M. A rapid stain for the direct microscopic examination of milk. *J. Lab. and Clin. Med.*, 14, 377-9. 1929.

<sup>2</sup>Mallman, W. L., and Churchill, E. A rapid test for determining bacteria in liquid eggs. *U. S. Egg and Poultry Magazine*, 48, 406. 1942.

Best results are obtained when the solution is held in a stoppered bottle and in the refrigerator when not in use. The solution should be filtered before using.

The ingredients employed by the writer in the above formula were as follows: ether, C. P., Bakers Analytical; methyl hydrate, Analar, (i. e., methyl alcohol) British Drug Houses Ltd.; methylene blue, 187717/88823, British Drug Houses Ltd; hydrochloric acid, Analar, British Drug Houses Ltd.

The milk slide is prepared in the usual manner by employing either a loop or a pipette. The smear is dried carefully and is then dipped into the staining solution for the required time, removed, washed, dried and examined. Staining for fifteen seconds or more will give a background which is completely decolorized. This may present a problem in focusing the microscope, especially when good quality milk having few bacteria is employed. Experience has shown that an exposure of from five to ten seconds will leave a faintly colored background on which it is easy to focus the microscope. The bacteria and other cells are stained blue.

It is realized that this method should be compared with other rapid staining procedures for the same purpose, such as those of Newmann and of North. As time is lacking at present for this comparison, the present preliminary note is published for the benefit of anyone who wishes to make use of the proposed method.

## A NEW CYTOLOGICAL TECHNIC, TANNIN-IRON III, FOR NUCLEOLI AND PLASTIDS

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Tannin-iron III, as a technic, differs very little from Salazar's tannin-iron I, and must be considered as a new modification of the latter<sup>1</sup>. But the results obtained are very different and its use in plant cytology will be found very advantageous due to its specificity for certain cellular organs.

This specificity arises from the previous hydrolysis in normal hydrochloric acid. The time of hydrolysis depends on the composition of the fixing fluids. In this way, the nucleoli and the "grana" of the plastids acquire a specific secondary tannophyly although they are not at first tannophylic.

The procedure is as follows:

I—*Fixation*. Any of the following fixing procedures may be used: Carnoy (2 vol. absolute ethyl alcohol, 1 vol. glacial acetic acid); salted formalin (10% formalin in 3% aqueous sodium chloride); "acetoforsaldier." (immersion for about 24 hours in 5% acetic acid; then for about 48 hours in salted formalin; rinsing for 24 hours in running water; and finally immersion for 3-6 days in 3% aqueous potassium dichromate).

II—Embedding in paraffin

III—Hydrolysis

1. Remove paraffin from sections and bring them down to distilled water.
2. Rinse in cold normal hydrochloric acid.
3. Hydrolyze in hot normal hydrochloric acid (58°-60° C.) for 4 minutes for material fixed in Carnoy's fluid and 2 minutes for material fixed in "acetoforsaldier." or salted formalin.
4. Rinse in distilled water.

IV—Staining

1. Transfer sections to Salazar's acetic tannin in which they must remain for 5 minutes.

(To prepare Salazar's acetic tannin, dissolve 6 g. tannic acid in 10 ml. glacial acetic acid and then add 30 ml. distilled water).

<sup>1</sup>See: Salazar, A. L. 1944. La technique de tannin-fer. *Stain Techn.*, 19, 181-5. Id. 1946. A new technic, tannin-iron II. Id., 21, 149-51.

2. Wash in distilled water.
3. Stain sections in 4% iron alum solution for about 5 minutes.
4. Wash thoroughly in running water.
5. Dehydrate and mount in Canada balsam.

## QUICK STAINING METHOD FOR FROZEN SECTIONS

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Since the advent and general acceptance of frozen sections in histological and pathological laboratories it has been necessary to devise methods for staining these sections. The usual method is fixing the tissue to a slide by the use of celloidin. This paper is an attempt to describe a permanent, quick method of staining frozen sections without distortion or mechanical tearing of the tissues.



FIG. 1. Spinal Ganglion cells. Rat.  $\times 450$ . Frozen section cut at  $10\mu$  and stained with Mayer's alum hematoxylin and eosin.

The equipment necessary for this procedure consists of a glass 'spot plate' and a perforated section lifter of such a size as to fit one of the depressions in the spot plate.<sup>1</sup>

<sup>1</sup>Section lifter used by writer (purchased from A. S. Aloe Co., St. Louis) has a bowl with a top diameter of 18 mm. and a 163 mm. handle soldered to one side of the bowl at right angles.

Almost all of the common stains can be adapted to this method. Naturally, those methods which depend upon impregnation cannot be included, although after staining by them the tissues can be sectioned by the freezing method.

#### PROCEDURE

The sections are cut from 5-15 $\mu$  and placed in distilled water. The stains and reagents of the particular method to be used are placed in the spot plate in the order in which they are to be needed. As an example the hematoxylin and eosin method will be described here.

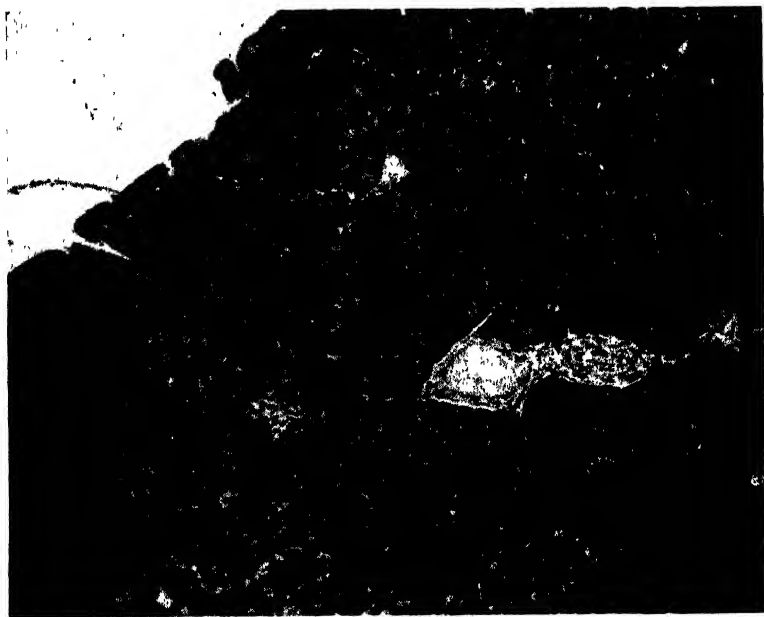


FIG. 2. Pancreas. Rat.  $\times 160$ . Frozen section cut at 15 $\mu$  and stained with Harris' hematoxylin and eosin.

In the first depression Mayer's alum hematoxylin<sup>2</sup> is placed. It requires but a few drops of solution to fill the depression. Following this the different concentrations of alcohol are placed in successive depressions. In this laboratory 25%, 35%, 50%, and 75% alcohol are used. In the following depression, 1% eosin in 75%

<sup>2</sup>Mayer's alum hematoxylin: Dissolve 1 g. hematoxylin in 1000 ml. distilled water. Add 0.2 g. sodium iodate and 50 g. ammonium or potassium alum. Add 1 g. citric acid and 50 g. chloral hydrate.

methyl alcohol is placed. This is followed by several changes of 95% alcohol and finally the clearing solution of creosote and beachwood oil is included.

A section of the tissue is picked up by the perforated section lifter and placed in the first solution in the spot plate. Because there is no embedding material in the tissue, staining is much more rapid than by other methods. In this laboratory it has been found that 15 seconds is long enough to stain the section, and 5 seconds is sufficient in each of the successive alcoholic solutions. Fifteen seconds in the clearing agent removes all excess stain and water.

In some cases the tissues are wrinkled after sectioning. This does not prevent staining and they will usually straighten out when placed in the cleaning agent. It is, however, sometimes necessary to straighten them by means of fine needles.

After clearing, the tissues are floated onto a clean glass slide and mounted in any of the common mounting media. Balsam is commonly used, but in this laboratory Clarite<sup>3</sup> has given better results.

Although the hematoxylin and eosin stain has been described it is possible to obtain consistent results with Mallory's triple dye stain, Masson's stain, and any of the single stains.

Sections made by this method of both fixed and unfixed tissues have given excellent permanent sections. Fig. 1 and Fig. 2 show tissues prepared by this technic.

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<sup>3</sup>Synthetic resin sold by Central Scientific Company, Chicago, Illinois.



## HISTOCHEMICAL LOCALIZATION OF ACID PHOSPHATASE IN GERMINATING MAIZE KERNELS

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**ABSTRACT.**—Sections of germinating maize kernels obtained by freezing technic were examined for acid phosphatase according to the histochemical method of Gomori. The embryonic axis, scutellum and aleurone layer were found positive for the enzyme. A microtechnical method for preformed phosphate, based on Sumner's colorimetric method for phosphorus, is described.

### INTRODUCTION

Save for the recent work by Glick and Fischer (1945a; 1945b; 1946) on phosphatases in wheat grains and sprouts, the application of the valuable technic of Gomori (1939; 1941) for the localization of phosphatases has been confined to studies of animal tissues. In the present investigation Gomori's method for acid phosphatase was adapted to a study of germinating maize kernels. The principle of Gomori's technic is as follows: Tissues are incubated with sodium glycerophosphate in the presence of lead nitrate. The phosphate liberated by the enzyme is trapped *in situ* by forming insoluble lead phosphate. The latter upon treatment with ammonium sulfide yields a brown to black deposit of lead sulfide, which affords a visual localization of sites of phosphatase activity.

### METHOD

Fully-matured maize kernels were induced to germinate and softened for sectioning by soaking them in water at 38° C. for 24 hours. The kernels were then trimmed and sectioned at once, or allowed to develop for an additional period between moist blotters at room temperature before further treatment.

Trimming was done with a razor-blade by cutting a longitudinal slice from each side of a kernel so that the remaining central portion, from which sections through the median longitudinal plane are to be obtained, contained the scutellum and embryonic axis. In this operation the kernels must be handled carefully, for the slightest rolling or crushing will be evidenced later in imperfect sections.

The trimmed kernels were sectioned with a sliding microtome, equipped with a freezing attachment. For gross demonstration of

acid phosphatase, sections of 100 to 120 $\mu$  in thickness were cut; while for cytological study sections of 60 to 80 $\mu$  were used.

Two methods of handling the sections were tried. In the first method the sections were transferred with a wet camel-hair brush to square cover slips (two sections were placed on each cover slip). Three drops of 95% alcohol were added over each section, then drained off, and replaced with absolute alcohol. The latter, in turn, was replaced with 1% alcohol-ether collodion. By exposure to the air for a few minutes, the collodion hardened; and the cover slips with the affixed sections were placed in the incubating mixture.

While the above method gave good results and is recommended in dealing with either soft or friable material, it was found that the sections of corn kernels were rigid enough to permit them to be transferred directly with a section-lifter through the reagents, which were contained in 25 ml. Stender dishes. In view of this, the freshly-cut sections were fixed in absolute acetone for 15 minutes, rinsed in distilled water, and placed in the incubating mixture. Sections held overnight, however, in ice-cold acetone and then incubated, showed no discernible loss in enzyme strength.

Incubation was carried on in an oven at 38° C. for 45 minutes. Since pure  $\alpha$ -sodium-glycerophosphate recommended by Gomori was not available, Moog's (1944) incubating mixture was used. It consists of the following ingredients mixed in the order given: acetate buffer at pH 4.7, 2½ parts; 5% lead nitrate, 1 part; distilled water, 15 parts; 2% sodium glycerophosphate (Eastman's 52% alpha and 48% beta), 3 parts. After standing overnight in a refrigerator the mixture was centrifuged to rid it of precipitated lead- $\beta$ -glycerophosphate, and the clear supernatant was used as the incubating mixture.

The incubated sections were rinsed in three changes of distilled water for 2 minutes. To remove lead-protein and lead-glycerophosphate they were next rinsed in 2½% acetic acid for 4 minutes. This was followed by a rinse in three changes of distilled water for 3 minutes.

The sections were then placed for 2 minutes in a dilute solution of ammonium sulfide (1 ml. of Eimer and Amend's light ammonium sulfide to 50 ml. of distilled water).

After rinsing in several changes of distilled water, followed by tap water, the sections were dehydrated through 50%, 95%, and two changes of 100% alcohol. The sections were retained in each alcohol for 5 minutes, except for the second change of absolute alcohol in which they were held for 10 minutes.

Next the sections were placed for 5 minutes in a 1:1 mixture of

xylene and 100% alcohol. They were cleared by keeping them 5 minutes in a first and 15 minutes in a second change of xylene. Finally the sections were transferred to slides and mounted in Clarite.

If desired the sections may be counterstained during the dehydration process. Eosin Y (0.5 g. in 100 ml. of 95% alcohol) gave satisfactory results.

Sites of phosphatase activity stain black or brownish-black. Likewise, because of chemical interaction with lead nitrate, any preformed deposits of phosphate will stain similarly. The preformed phosphate will appear if glycerophosphate is omitted from the incubating mixture, while sites of phosphatase activity will not. Therefore control sections were treated like the experimental ones except that glycerophosphate was omitted from the incubating mixture. Thus control sections showed preformed phosphate while the experimental ones showed in addition phosphate liberated from the substrate by the enzyme. Slides prepared as described above showed no appreciable fading after three months.

The distribution of preformed phosphate in the control sections was found to be in agreement with that obtained by a microtechnical test for inorganic phosphate, suggested by Sumner's (1944) method for the colorimetric determination of phosphorus. With a razor-blade, thick free-hand sections were made of corn kernels. After 15 minutes fixation in absolute acetone, the slices were placed in a 1% ammonium molybdate solution for 15 minutes to allow the inorganic phosphate in the tissues to combine with the molybdate to form phosphomolybdic acid. Following a rinse in distilled water, the sections were examined with a dissecting microscope while immersed in a freshly prepared, acidified ferrous sulfate solution (5 g.  $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$  and 5 ml. of 7.5 N  $\text{H}_2\text{SO}_4$  made up to 50 ml. with distilled water). The blue-colored areas that appear within 5 minutes are caused by a reduction of phosphomolybdic acid to blue phosphomolybdous acid. Since the blue stain diffuses, the examination must not be delayed.

## RESULTS

Acid phosphatase occurs in all parts of the embryonic plant and in the aleurone layer (Fig. 1). It is not present in the pericarp, nor is it found in the endosperm except in regions contiguous to the epithelial layer and to the aleurone layer.

*Embryonic axis*—In the plumule, coleoptile, radicle, coleorhiza, and hypocotyl, the cytoplasm, as indicated by small brown-staining granules, has moderate amounts of acid phosphatase; the nuclei, however, are strongly positive. Control sections of the embryonic

## EXPLANATION OF FIGURES

<i>Al</i> , aleurone layer	<i>Ep</i> , epithelial layer
<i>Ea</i> , embryonic axis (radicle indicated)	<i>P</i> , pericarp
<i>En</i> , endosperm	<i>Sc</i> , scutellum
<i>SCS</i> , storage cells of scutellum	

Fig. 1. Illinois Hull-less, popcorn. Germinated 50 hours, 100 $\mu$ . Stained for acid phosphatase.  $\times 5$ .

Fig. 2. Hybrid Golden Bantam, sweet-corn. Germinated 24 hours, 60 $\mu$ . The storage cells of the scutellum and the epithelial layer are positive for acid phosphatase.  $\times 290$ .

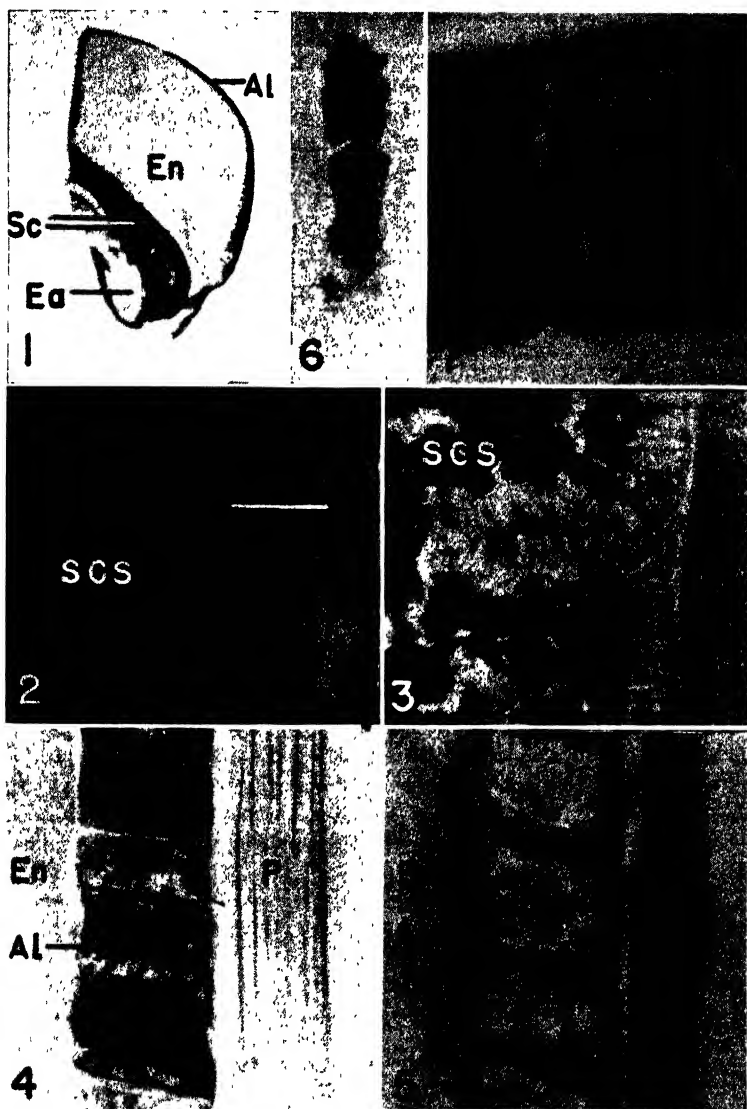
Fig. 3. Hybrid Golden Bantam, sweet-corn. Germinated 24 hours, 60 $\mu$ . The storage cells of the scutellum are positive for preformed phosphate, but the epithelial layer is negative.  $\times 290$ .

Fig. 4. Hybrid Golden Bantam, sweet-corn. Germinated 50 hours, 80 $\mu$ . The aleurone cells are positive for acid phosphatase.  $\times 290$ .

Fig. 5. Hybrid Golden Bantam, sweet-corn. Germinated 50 hours, 80 $\mu$ . The aleurone cells are weakly positive for preformed phosphate.  $\times 290$ .

Fig. 6. Hybrid Golden Bantam, sweet-corn. Germinated 24 hours. The epithelial cell shows cytoplasm, nucleus, and nucleolus positive for acid phosphatase.  $\times 1750$

Fig. 7. Hybrid Golden Bantam, sweet-corn. Germinated 50 hours. The aleurone cells show the cytoplasm, irregularly-shaped nuclei, and nucleoli positive for acid phosphatase.  $\times 600$ .



axis reveal a slight quantity of preformed phosphate, and it is found only in the cytoplasm.

*Scutellum*—In the storage cells of the scutellum, acid phosphatase is strongly marked by heavy black deposits in the cytoplasm. The epithelial layer of the scutellum is also positive and black cytoplasmic granules are most pronounced in the ends of the cells facing the endosperm (Fig. 2). In sections of  $100\mu$  or more in thickness a dark zone in the endosperm bordering the epithelial layer suggests a diffusion of the enzyme from the epithelial layer. The nuclei of the storage cells and of the epithelial cells of the scutellum are positive; the nucleoplasm takes a diffuse brown stain, and a single nucleolus appearing in each nucleus stains black (Fig. 6).

Control sections show considerable deposits of preformed phosphate in the cytoplasm of the storage cells of the scutellum. The epithelial layer, however, is negative for preformed phosphate (Fig. 3). The aforementioned figure shows one of the characteristic invaginations of the epithelial layer into the storage tissue of the scutellum.

The abundance of acid phosphatase in the storage cells of the scutellum may be related to the abundant lecithin stored there. Glycerophosphate, resulting from the hydrolysis of lecithin, would serve as the normal substrate for the enzyme. The liberated phosphate, upon translocation to the meristematic tissue of the embryonic plant, would enter into the formation of nucleic acid and nucleoproteins.

*Aleurone layer*—The heavy-walled cells of the aleurone layer are strongly positive for acid phosphatase (Fig. 4). Black granules are present in the cytoplasm; and within the dark-staining nucleoplasm of each cell, a jet-black nucleolus is present (Fig. 7). In sections of  $100\mu$  or more in thickness, a dark stain in the peripheral zone of the endosperm indicates a diffusion of the enzyme from the aleurone layer.

Preformed phosphate is apparent in minimal amount in the cytoplasm in control sections of the aleurone layer (Fig. 5).

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## THE ACETOCARMINE SMEAR TECHNIC<sup>1</sup>

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**ABSTRACT.**—The acetocarmine smear technic is described primarily for beginners. An effort was made to bring together into one paper the work-a-day details to be followed in collecting specimens and making preparations of plant chromosomes at prophase, of condensed chromosomes at meiosis and mitosis (including mitosis in microspores), and of coiled chromonemata. A schedule for converting temporary slides into permanent mounts is given, and a technic for making smears of the chromosomes in the salivary glands of *Drosophila* is described.

### INTRODUCTION

It is the hope and intent of the writer to prepare a paper with sufficient information to enable a novice to develop proficiency in preparing acetocarmine smears by following the instructions. However, the beginner should not be too easily pleased or displeased by the results of early efforts. The technic is something of an art, and as such requires patience and continued effort to equal or surpass the results of the masters of the art. It is also hoped that the paper may be helpful to those already using the technic, either in their own work or as a reference to which they can direct students.

Current acetocarmine smear procedures are based on the method as developed by Belling (1926) and improved by McClintock (1929 and unpublished), and others. The technic has been used successfully on a wide variety of plant and animal material, such as anthers, root tips, embryonic leaves, buds, salivary glands of *Diptera* (Painter, 1934), various embryonic tissues of insects (Carlson, 1942; Smith, 1943), and testes and embryonic tissues of birds and mammals (Painter, 1939). Descriptions of the technic as it is applied to certain species are given herein. Adaptations to additional species may be found in the references (which were selected more for details on

<sup>1</sup>Published as Scientific Paper No. 687, College of Agriculture and Agricultural Experiment Stations, Institute of Agricultural Sciences, State College of Washington, Pullman, Washington.

The author is indebted to Dr. A. B. Griffin of the Department of Zoology, University of Missouri for the section on smears of salivary gland chromosomes in *Drosophila*. He also desires to acknowledge indebtedness to Drs. C. R. Burnham and Barbara McClintock from whom he learned the technic, and to these and Drs. H. C. Aase, C. L. Huskins, W. E. Maneval, Karl Sax, and others for suggestions incorporated in the manuscript.

technic than for precedence) and in other papers in the literature, many of which were cited by Darlington and La Cour (1942).

### GENERAL INFORMATION AND INSTRUCTIONS

There is considerable variability among carmine dyes. The National Aniline and Coleman and Bell carmines have been tested and recommended.

Acetocarmine is prepared by boiling an excess of carmine in 45% acetic acid (about  $\frac{1}{2}$  gram in 100 ml.) for 2 to 10 minutes or in a reflux condenser or stoppered bottle for  $\frac{1}{2}$  hour or more. After cooling, the acetocarmine is filtered and used full strength for staining pachytene preparations but may be diluted with 45% acetic acid for condensed chromosomes. Thomas (1940) recommended using 1 part stain to 2 parts of 45% acetic acid for chromosomes of fruit trees. Sometimes it is necessary to dilute or refilter old batches of stain. A good batch seems to be a matter of luck, and one just continues to make stain until a product that gives satisfactory staining is obtained. A good stain, properly used, results in darkly stained chromosomes with almost clear centromeres, and nearly colorless cytoplasm. Once obtained, a good stain remains good for years.

Care must be exercised in the amount of stain used in preparing smears. Pachytene preparations of maize (and related plants) are usually better if the drop of carmine is of such size that it barely reaches the edges of the cover slip. The stain draws the cover slip down closely to the slide, flattening the large microsporocytes without breaking the chromosomes. On the other hand, slides of condensed chromosomes are usually better if prepared with a larger volume of stain and the excess pressed out on a blotter, simultaneously flattening the cells and spreading the chromosomes. In examining newly made slides of root tips it is particularly important to have enough stain under the cover slip so that effective flattening of individual cells with pressure from a needle can be accomplished.

Additional flattening of individual cells (especially important for photomicrography) may be obtained by applying pressure to the cover slip over the cell for a day or longer. This pressure can be applied by placing a small piece of paper over the cell and weighting it down with weights, or by applying pressure with a spring-type clothespin.

If slides are to be examined later, they may be sealed around the edges of the cover slip with a sediment-free mixture (equal parts) of gum-mastic and hard paraffin. Paraffin alone will do but not so well. A satisfactory device for applying the seal can be made from a wire test tube holder or from a piece of copper wire. Another, water-

soluble seal of the following (or similar) composition has been used by various workers:

Distilled water	90 ml.
Glycerin	16 ml.
Gum arabic	50 g.
Chloral hydrate	100 g.

H. C. Aase and M. Owenby have used this seal in the dry climate of Pullman, Washington, and found that many slides remain usable for a year or longer. The stain is allowed to evaporate (if necessary) until it barely reaches the edges of the cover slip; then the seal is applied along the edges of the cover with a medicine dropper. A weight (*e. g.* a bolt nut) is placed on the cover slip to prevent the seal from flowing under it.

For many purposes, slides can be examined, even using the oil immersion objective, immediately or within a few hours after preparation and discarded without sealing.

Good temporary smears usually improve for a day or longer (although poor slides get worse) and may remain good for a month or more. Therefore, it is often desirable to wait a day or so before examining a slide thoroughly, before making the mount permanent, or before taking photomicrographs.

Storing slides in a refrigerator is not satisfactory because frequently contraction breaks the seal, allowing the smears to dry up.

The spindle is often not clear in acetocarmine preparations, but may be fairly apparent in material fixed for a long time or in slides that are old.

Nucleoli are reasonably clear in acetocarmine smears.

Aceto-orcein (1 gram orcein dissolved in 45 ml. of hot glacial acetic acid, and diluted when cool with 55 ml. of distilled water) can be used in the same way as acetocarmine (La Cour, 1941) and for some materials has certain advantages. It is not so likely as acetocarmine to overstain the chromosomes and does not stain the cytoplasm or nucleoli.

Perhaps it should be mentioned that especially satisfactory results are obtained in viewing and photographing acetocarmine smears if green or yellowish-green light filters (such as Eastman Wratten "M" Filters No. 58 and 56, alone or in combination, or No. 62 for maximum contrast) are used.

#### FIXATIVES AND FIXATION

Some materials may be satisfactorily fixed and stained with acetocarmine alone, but for most purposes better preparations are obtained if the material has been previously fixed. Fixation may vary

from a few minutes to several days depending upon the material, fixative, temperature, and purpose for which the slide is to be made. Fixation of condensed chromosomes is not so critical as fixation of prophase chromosomes and may be continued longer without serious loss of quality. The fixatives usually used are:

Farmer's fluid (freshly mixed)		
95% Ethyl alcohol (or absolute)		3 parts
Glacial acetic acid		1 part

Carnoy's fluids (freshly mixed)		
	A	B
95 Ethyl alcohol (or absolute)	6 parts	6 parts
Chloroform	3 parts	1 part
Glacial acetic acid	1 part	3 parts

Farmer's fluid is used for pachytene preparations of maize, sorghum, etc. Carnoy's fluid A (some workers prefer B) gives better preparations of the condensed chromosomes of wheat and many other species. It does not harden the cells so much; leaves the cytoplasm clearer; does not swell the chromosomes so much; and keeps the specimens usable longer than Farmer's.

There is considerable evidence that better fixation, resulting in less clumping of chromosomes, may be obtained if the specimens are small. Thus, in some difficult materials, better results may justify fixing individual anthers or making hand sections of the larger tissues.

Emsweller and Stuart (1944) described a method for obtaining better preparations of meiosis in plants with large or numerous chromosomes by the use of enzymes after fixation. They washed out the fixative and treated the material for 10 minutes to several hours in a 1% solution of Clarase (or other enzyme extracts) before staining, and obtained very promising results.

In taking specimens it should be remembered that in some species there are definite daily cycles of divisions. These cycles are not clear-cut in the cereals and many other plants, but material (microspores particularly) fixed a little before mid-day, or possibly earlier for meiosis, seems to have more nuclei undergoing division than material fixed at other times.

If the specimens are not to be used when properly fixed, they may sometimes be kept usable (up to a year or longer) if stored at below freezing temperatures in a refrigerator, and allowed to warm up for several hours before smears are made. The specimens can be transferred to 70% alcohol, but storing immediately after killing in the original fixative seems to be about as satisfactory. In neither case are the slides ordinarily as good as those obtained from freshly fixed specimens.

## PROCEDURES FOR SPECIAL PURPOSES

## MEIOSIS

A. *Maize (teosinte, sorghums, etc.)*. Specimens are taken about 2 weeks before pollen shedding would occur. The tassel, as felt through the layers of sheaths and leaves, should not feel hard (too young) or soft (too old) but like an inflated football—resilient. A 2- to 4-inch, vertical slit, barely to the tassel, is made with a razor blade, and 2 or more intact branches are removed with forceps. If it is desired that the tassel grow on to maturity, the wound should be carefully closed and covered with a band of gummed paper tape.

The tassel branches are fixed in 5 ml. or more (barely covered) of Farmer's fluid for 1 or 2 days. If fixed in 2:1 or 1:1 instead of 3:1, slides may be prepared sooner. The quality of the smear is strongly dependent on the proper degree of fixation. For smears of pachytene, the material is easily fixed too long for best preparations, and if not to be worked within 2 or 3 days should be stored in a refrigerator.

After fixation, smear preparations may be made as follows:

1. Place a branch of the tassel in a small quantity of the fixative in a petri dish (over black to improve visibility).
2. Dissect out one of the anthers in a spikelet and place it on a clean, dust-free slide. Fine-pointed iron or stainless steel dissecting needles (thoroughly cleaned in a drop of acetocarmine at the beginning of each slide-making session to remove rust and dried stain), with  $\frac{1}{8}$ -inch of the end curved at about a right angle may be used.
3. Add a small drop of acetocarmine to the anther at once.
4. Cut the anther in two and gently press it (beginning at the uncut ends) with the curved point of a needle, forcing the pollen mother cells out.
5. Briefly examine the sporocytes for the proper stage under the low power of the microscope. For the beginner, it is desirable to put on a cover slip for more thorough study, but he should soon strive to recognize the stages in the uncovered drop.
6. If the desired stage is present, tap out more of the pollen mother cells, carefully remove the debris, and quickly put on a clean cover slip (previously readied). A  $\frac{3}{8}$ "-square, No. 1 cover slip is usually recommended. If too much time is taken to determine the proper stage, crystals will form at the edges of the stain and the preparation is spoiled, unless the crystals can be dissolved with more stain. If the proper stage is not present, the drop of acetocarmine containing the anther can be wiped off with cheese-

cloth and an older or younger anther selected for smearing on the same slide.

7. Heat the preparation (over an alcohol lamp, gas burner, or electric light bulb) to near the boiling point a few times. Be careful not to expand the air bubbles unduly, and examine the preparation under the microscope after each heating for proper staining. If a speck of dust, etc. holds the cover slip up at one spot, local pressure with a dissecting needle (using care not to move the cover slip) may remedy the trouble.
8. After the mount has cooled, carefully seal.

In learning the technic, it is necessary to strive for speed. Later, when proficient, it is possible to work so fast that the staining may not be dark enough.

**B. *Wheat (and related plants).*** The whole spike is fixed just before the tips of the awns (in awned types) appear out of the boot of the last leaf; or when the sheath of the last leaf has emerged about half way. Fixation is in Carnoy's fluid for 2 to 5 days. The slides are prepared in the same way as recommended for maize, except usually all 3 anthers from a floret are used to make one slide, and a larger drop of stain is used.

**C. *General.*** After the technic has been mastered for a particular species, the problem of finding the proper stage for taking specimens and where the most advanced and latest anthers are to be found in the specimen usually presents no serious difficulty. However, in plants such as oats, where a regular series of stages is not evident, finding whether the specimen has anthers at the proper stage is laborious at best.

#### ROOT-TIP SMEARS

Farmer's or Carnoy's fluid, acting for 2 to 10 days, or even longer, gives good fixation.

1. Place about 1 mm. of the root tip on a slide and macerate in a small drop of acetocarmine with a spear-point needle or glass rod.
2. Add a drop of stain, cover (without prior examination under the microscope) and heat.
3. Flatten individual cells by applying pressure with the point of a dissecting needle while the cell is observed under the low power of the microscope.
4. Reheat, cool, and seal.

Some workers prefer to make thin hand-sections of the root tips with a razor blade before smearing. That may be helpful with larger root tips such as those of maize.

It is very important to get root tips in which cell division is going on rapidly. Thus, it is desirable, where possible, to take root tips from seeds just starting to germinate on blotters, because they are growing rapidly and are free of dirt. If tips are taken from plants growing in the soil, they must be carefully freed of grit (*e. g.*, with the fingers or lips) before fixing or smearing. Judicious flattening of selected cells in newly prepared slides under observation in the microscope is also important.

Pretreatment with paradichlorobenzene (Meyer, 1945) greatly improves the smear technic for chromosome counts and for studies of chromosome morphology (Fig. 1). The method as described above would be modified only to the extent of soaking the root tips (or

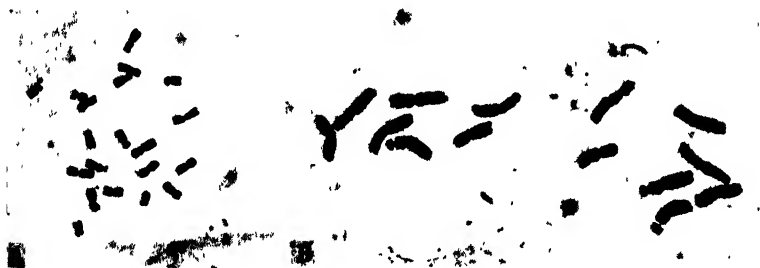


FIG. 1.\* Somatic chromosomes from root tips pretreated for 2 hours at room temperature in a saturated aqueous solution of paradichlorobenzene (Meyer, 1945), fixed in Carnoy's fluid and stained with acetocarmine. Counting of the chromosomes is facilitated, and morphological characteristics are accentuated A. Maize B. Barley ( $\times 850$ ).●

\*The writer is indebted to Dr. J. G. O'Mara for the use of Fig. A.

other embryonic tissue) in a saturated, aqueous solution of paradichlorobenzene for 1 to 4 hours before fixing. According to Meyer the solution is "prepared by mixing about 5 or 10 g. paradichlorobenzene crystals and about 500 ml. distilled water in a stoppered bottle and holding at about 60°C. overnight. It is used at room temperature." It should also be possible to adapt the method for microspore divisions where the material is more favorable for study of chromosome morphology.

With some species better smears of root tips are obtained if the middle lamella holding the cells together is digested so that the cells separate freely. This has been accomplished by pretreatment of the fixed tissue with 10% HCl for 10 to 30 minutes (and returning to the fixative) prior to staining, and in various other ways (Warmke,

1935). Faberge (1945) recommended the use of digestive juices from the stomachs of snails for the same purpose.

#### MICROSPORE DIVISIONS

In maize, wheat, barley, and rye, specimens may be fixed for the first mitotic division in the pollen grain about 5 to 7 days after meiosis—at the stage the tassel (in maize) is emerging and starch is beginning to appear in the spores. The second division occurs 5 to 7 days later, after a considerable deposit of starch is present. Some of the starch may be “bleached out” by adding crystals of chloral hydrate to the acetocarmine (3–10 grams to 1 ml.). Some chloral hydrate is almost necessary for clear observations on the second division. However, if too much chloral hydrate is added, the chromosomes are fragmented and destained. Frequently, particularly for the second division, it is difficult to determine whether the proper



FIG. 2. Root-tip cell of barley. No pretreatment.

stage is present without putting on a cover slip and heating the slide.

Sax's method of staining microspore divisions in *Tradescantia* is to smear the material on a dry slide and fix in Farmer's fluid for 10 minutes. The fixative is then drained off and replaced with several drops of acetocarmine. A cover slip is put on, the mount heated, set aside for 10–15 minutes, reheated, pressed firmly under a sheet of bibulous drying paper (Cenco), cooled, and sealed.

In species in which nuclear divisions occur in the pollen tubes, acenaphthene and colchicine were successfully used by Swanson (1940, 1942) for facilitating the study of chromosomes.

#### STAINING TO SHOW COILS

Chromonemata coils in contracted chromosomes may be stained effectively in unfixed specimens.

1. Place a flower of the material under study, *e. g.* a spike of rye, on a moist blotter in a petri dish; it will stay fresh for several hours.
2. Take part of an anther and find the proper stage in the usual manner by examining a few pollen mother cells in a drop of acetocarmine.
3. If the proper stage is present, press and carefully draw the remainder of the anther (or another anther from the same floret) along a clean slide leaving ribbons of sporocytes.



FIG. 3. Desk equipped for the aceto-carmine smear technic.

*Left:* microscopic equipment.

*Right, front:* Smearing apparatus.

*Right, back:* Apparatus for making permanent preparations.

For close-up of the right side of desk, see Fig. 4.

4. Pretreat the smear for 30 seconds or less in a solution of ammonia water in 30% alcohol (3 to 8 drops of concentrated ammonium hydroxide to 50 ml. of 30% alcohol).
5. Add a drop of acetocarmine.
6. Cover.
7. Heat, cool, and seal.

The pretreatment may be done in a staining jar or by putting a few drops of the solution on the slide as it lies on a table, removing the excess with blotting paper, held at the edge of the drop, or pouring it off. The manipulations must be done very carefully, so as not to damage the chromosomes.

If step 4 is omitted, ordinary acetocarmine smears from unfixed material are obtained.

The above method of staining to show coils was developed by Sax and Humphrey (1934), and was based on the discovery by Kuwada and Nakamura (1934) that ammonia accentuates the coiled structure of condensed chromosomes. Matsuura (1938) reported obtaining satisfactory slides for the study of coils merely by pretreating the

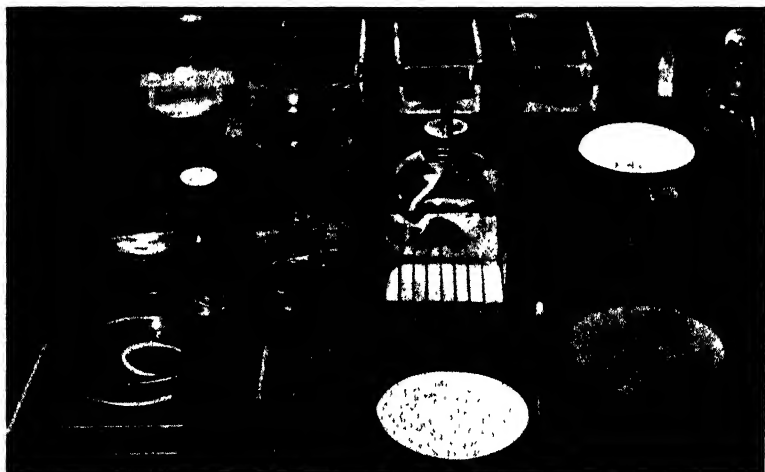


FIG. 4. Equipment used in making acetocarmine smears of plant tissues (front) and converting temporary to permanent slides (back row). In the front row are: 2 dissecting needles and a spear-point needle, slide, petri dish and specimen over black paper, forceps, blotter, glass-marking pencil, seal and applicator. For dry climates, at least, the water-soluble seal mentioned in the text is preferable. Second row: specimen bottle, acetocarmine, cover slip holder. Third row: cover slips and slides in jars containing alcohol, alcohol lamp, a sealed slide with lead weights to flatten an individual cell. Back row: a petri dish with a U-shaped glass rod supporting a slide, razor blade, 3 staining dishes, a slide with a clothespin pressing out excess balsam, balsam.

smears for  $\frac{1}{2}$ -2 minutes in tap or distilled water. Boiling water, desiccation, and cyanides have been recommended as helpful in the study of coils also.

#### MAKING ACETOCARMINE SMEARS PERMANENT

If it is desired to convert temporary slides into permanent ones, the method of McClintock (1929) or Buck (1935) may be used, producing slides that will remain good for years, and probably in-

definitely. The following method of Sears (1941) gives excellent results with smears of maize, wheat, and barley.

1. Gently chip off the temporary seal with a razor blade. More of the seal can be removed with a small camel's hair brush wet with xylene (or 45% acetic acid for the water-soluble seal).
2. Soak the mount in equal parts of glacial acetic acid and 95% ethyl alcohol, until the cover slip comes off. (Process may be hastened by adding a little tertiary butyl alcohol to the solution.) Placing the slide upside down on a U-shaped glass rod in a petri dish (or in a porcelain dish with two ridges on the bottom) containing the solution is convenient.
3. Treat with equal parts of 95% ethyl alcohol and tertiary butyl alcohol for 2 minutes or longer, transferring the slide and cover slip to the staining dish containing the new solution and keeping them in the same relative positions. A split wooden match has been recommended as a convenient handle for transferring the cover slip.
4. Tertiary butyl alcohol, 1 or 2 changes, 2 minutes or longer in each.
5. Drain slide and cover slip (edges on a blotter) momentarily.
6. Apply a small drop of fairly thin balsam to slide and quickly reassemble slide and cover slip in same position as before. Press out excess balsam on a blotter.

Another method that is commonly used with smears of both plant and animal tissues is to follow steps 1 and 2 above, then run the slide and cover slip through 2 changes of 95% or absolute alcohol and mount in Euparal or Diaphane.

It is sometimes advisable to apply pressure to the cover slip for several days to force out the excess balsam. This can be done by weights or preferably by means of a spring-type clothespin that will hold the cover slip in place. A piece of glass, sheet metal, or stiff strap iron (slightly smaller than the cover slip) under the clothespin will equalize the pressure.

A considerable number of slides (50 or more) can be made permanent using the same solutions in Coplin jars, or preferably in staining jars in which the slides rest on edge, without replacing or replenishing. However, the alcohol-acetic-acid solutions should be mixed just before they are used.

More material will be retained on the slide and cover slip, and because of the residues of stain, it is easier to remount the cover slip in the original position, if the preparation is several days old before

it is made permanent. If the cover slip is inadvertently broken, the pieces can be remounted with reasonable satisfaction.

If the material, such as microspores, is likely to be lost in the process of making the slide permanent, the cover slip or slide may be coated with a thin layer of Mayer's egg albumen (possibly Haupt's Adhesive would be better) before the smear is made. Another possibility is to lay the slide and cover slip flat on a table, putting the solutions on with a medicine dropper and taking them off with blotting paper, held at the edge of the drop, or pouring them off.

If the preparation has become too darkly stained before being made permanent, it may be destained by running 45% acetic acid under the cover slip and heating.

A number of methods have been devised to prepare smears that are more or less permanent, thus eliminating the necessity of converting temporary mounts. Two of these involve the use of dioxane and white corn syrup (Hillary, 1938, 1939). Another involves the use of gelatin or other media (Zirkle, 1940). These methods are satisfactory for some materials but are not as widely applicable as the temporary smear technic.

#### SALIVARY GLAND PREPARATIONS OF DIPTERA

The preparation of salivary gland smears from Dipteran larvae (*Drosophila*, *Sciara*, *Chironomus*, *Simulian*, and others) is a common application of the acetocarmine smear technic. The procedure described here is recommended particularly for *Drosophila*; modifications required by other genera will suggest themselves to the investigator as he works.

An agar culture medium of any standard high-yield type (high brewer's yeast content) is used as the substrate for 2 to 6 pairs of adult flies. The adults are transferred to fresh culture vials at 6-hour intervals in order that an array of larvae of different ages may be available. Cultures are maintained at the standard temperature of 70-72°F. Larvae are ready for dissection when they emerge from the food mass and begin to crawl upon its surface or upon the sides of the container in preparation for pupation. As with pachytene preparations of maize, there is considerable variation among stocks as to the quality of smear preparations obtained.

1. With a small wire loop, transfer active larvae to a black dissecting dish containing 0.07% NaCl.
2. *Dissection.* Using two pairs of fine-pointed forceps, grasp the most anterior tip (oral hook region) of the larva with one hand and the posterior end of the body with the other. A

slight pull immediately separates the head region from the rest of the body, trailing out the intact salivary glands and the anterior portion of the alimentary canal. Maintaining a firm grip on the oral hook fragment, isolate the glands by breaking their common duct with the second pair of forceps, keeping the pair of glands united. Practice will result in accuracy and speed in dissection; note that these qualities cannot be achieved through the use of dissecting needles in place of forceps.

3. *Staining and fixing.* After several pairs of glands have been dissected, they may be transferred to a small dish of acetocarmine with a medicine dropper. The time required for staining varies markedly with different species and may be estimated only after experimentation. *D. melanogaster* ordinarily requires a staining period of 20 to 30 minutes.
4. *Smearing.* With a pipette, transfer a drop of acetocarmine and several pairs of glands to a clean slide; drop a cover glass on without regard for air bubbles, which will be eliminated during the smearing process, and blot the covered slide gently with filter paper. Under a dissecting microscope, press out the glands with a blunt needle, using sharp, light taps to rupture isolated cells. The desired degree of spreading is readily observed at a magnification of 30 $\times$  or greater, since the salivary chromosomes are easily visible.
5. *Temporary mounts.* Seal, if the mount is to be used as a temporary slide.
6. *Permanent mounts.* Place fresh slides (or slides whose seal has been chipped off) in the vapor of 95% ethyl alcohol and allow 6 to 8 hours of vapor-dehydration as recommended by Bridges (1937). A small desiccator with alcohol below a slide-bearing shelf, or a Stender dish (lid sealed with vaseline) containing 5 ml. of alcohol may be used as the dehydrating vessel. After the dehydration period, the slides are immersed in 95% alcohol and may be allowed to remain for 1 to 12 hours. At the end of the immersion period, the slides are placed flat upon a paper towel without removal of excess alcohol. A drop of Euparal or Diaphane is placed at one side of the cover glass and is caused to flow gently under the cover by careful raising of the cover with a sharp needle; the opposite side of the cover should be held in position with the fingers during the process, since sliding of the cover tends to roll up and spoil the thin smear. To complete the mount, a drop of Euparal is placed at opposite edges

of the cover slip. The Euparal will be drawn under the cover slip as the alcohol evaporates, preventing air bubbles.

#### WASHING SMEAR SLIDES

Acetocarmine smear slides may be cleaned efficiently as follows:

1. Remove the paraffin-gum-mastic seal or immersion oil by soaking the mounts in xylene for about a week. The water-soluble seal recommended can be removed by soaking in water.
2. Separate the cover slips from the slides (a razor blade will be helpful in some cases.)
3. Wash the cover slips and slides separately in warm, (not hot) soapy water. Use care not to scratch the slides against one another.
4. Rinse in running water.
5. Store in 70% alcohol until used.

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## LABORATORY HINTS FROM THE LITERATURE

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A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOK AND PAPERS FROM OTHER JOURNALS  
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

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### BOOK REVIEW

COOPER, HERBERT J. (Editor). **Scientific Instruments.**  $5\frac{1}{2}$  by  $8\frac{1}{2}$  inches. 305 pages. Illus. Chemical Publ. Co., Brooklyn. \$6.00. 1946.

This book discusses a wide range of instruments, used in the laboratory, in the field, and in industry, for making physical measurements, not only describing the instruments themselves but explaining the fundamental principles on which they are based. The book is profusely illustrated with diagrams and photographs, and the language is clear and sufficiently non-technical so that one does not need to be a specialist in the field covered by any particular chapter in order to understand it. It is, perhaps, hardly a book for the layman; but it is certainly well designed to give any scientist a fair comprehension of the instruments used in fields of work other than his own. Fifteen collaborators are named as having contributed to the work; in fact it is obvious that no one scientist, without such collaboration, could have written intelligently about the instruments used in such a variety of different fields. Nevertheless, the style is so consistent throughout, as to suggest one hand doing most of the actual writing; presumably it is largely modesty that has inspired the use of the term "editor" on the title page.

The book is divided into five sections: Optical Instruments; Measuring Instruments; Navigational and Surveying Instruments; Liquid Testing; Miscellaneous (including calculating machines, vacuum tubes, etc.) The editor and all his collaborators are British; and it is often of interest to the American reader to note differences between the British approach to such subjects and that with which he is more familiar in the United States. Possibly the omission of one or two types of instruments (e.g. spectrophotometers) may be explained by the fact that different instruments are in use in the two countries.

Of special interest to readers of STAIN TECHNOLOGY is, naturally, the section on Optical Instruments, particularly the chapter on The Microscope and Electron Microscope. These subjects are adequately, although of necessity briefly, treated; the few pages on the electron microscope, for instance, can be recommended to anyone, even though unfamiliar with electronics, who wishes to obtain an understanding of this new and rather complicated instrument.—*H. J. Conn.*

### MICROSCOPE AND OTHER APPARATUS

KINGMA BOLTJES, T. Y. (U. Amsterdam, Holland.) **Some experiments with blown glasses.** *Antonie van Leeuwenhoek, J. Microbiol. Serol.*, 7, 61-76. 1941.

Experiments were carried out with blown glass spheres in order to ascertain whether the observations reported by van Leeuwenhoek might be made by means of these. Microphotographs clearly show that this is actually the case. Van Leeuwenhoek, however, mentions his use of ground lenses. At low magnification these are certainly better than blown glasses. The possibility of van Leeuwenhoek having used cover slips is discussed.—*M. P. Lohnis.* (Courtesy *Biological Abstracts*).

SHRYOCK, A., and ASHLEY, L. M. **Staining racks of three designs for coverglass preparations.** *J. Tech. Methods*, 25, 63-6. 1945.

Staining racks in which up to sixty cover-glass smears can be safely stained at once are described. The racks are made of stainless steel which is resistant to most staining reagents. They measure approximately 68 mm.  $\times$  27 mm.  $\times$  24 mm. Smaller racks suitable for student use are also described.

The use of these racks conserves time, space, and reagents.—*C. Randall.*

VICKLUND, R. E. Preventing the fungus fouling of optical instruments. *Ind. Eng. Chem.*, **38**, 774-9. 1946.

Metallic foil containing 15  $\gamma$  of radium per sq. in. in equilibrium with its decay products prevents fungus fouling of optical instruments. Lenses up to 3 in. in diameter can be adequately treated by surrounding each lens surface with a strip of foil, the width of which is equal to one-ninth of the lens radius. The use of radium-activated foil is economically feasible and presents no health hazards.—R. T. Whittenberger.

#### MICROTECHNIC IN GENERAL

BOURDON, H. Nouveau procédé d'inclusion par les résines polyvyniliques. *Bull. d'Histol. Appl.*, **20**, 46-8. 1943.

The author describes a new method of embedding with impregnation by vinyl acetate which solidifies by polymerization to a hard, colorless mass. Spontaneous polymerization may be avoided by adding 5% hydroquinone to the stock solution. The hydroquinone must be removed by distillation before using.

The recommended procedure is as follows: Fix in formalin, sublimate or a bichromate mixture. (Picric acid interferes with the polymerization reaction.) Dehydrate in four changes of dioxane over a period of 24 hr. Impregnate for 24 hr. with two changes of pure vinyl acetate. To prepare the embedding fluid, add 3% benzoyl peroxide to the vinyl acetate and boil to a sirupy consistency. Pour into a cellophane mold and add the impregnated tissue. Place in an oven at 37° C. for 48 hr., then at 56° C. for 48 hr. After cooling, immerse in water and remove the mold. The block may be mounted on a wooden base with the aid of a little melted cellophane, and sections may be cut as thin as 2 or 3  $\mu$ . Before staining, the embedding material may be dissolved in an organic solvent such as toluene.

The advantages of this method are that very thin sections may be obtained without tearing or distorting the tissue and that the sections adhere remarkably well to the slide. The disadvantages are the long and complicated procedure and the inability to obtain ribbons.—Jean E. Conn.

CARES, R. A note on stored formaldehyde and its easy reconditioning. *J. Tech. Methods*, **25**, 67-70. 1945.

Methods for overcoming the tendency of formaldehyde stock solutions to form paraformaldehyde by polymerization and formic acid by oxidation are given. To recondition formaldehyde which has become weakened due to polymerization the author recommends the following procedure: shake milky solution to suspend sediment; pour into Mason jars and seal tightly; autoclave at 15 lb. for 30 min.; cool. A water-clear solution results which may be stored or diluted for use.

Dilute (10%) solutions tend to form acid more readily than stock solutions (approx. 40%). Marble chips are useful for neutralizing diluted solutions.—C. Randall.

DELAUNAY, A. La déshydration méthodique dans la technique histologique. *Bull. d'Histol. Appl.*, **17**, 246-8. 1940.

The conventional methods of dehydration call for the tissue to be placed in the bottom of a vessel containing alcohol. Since water is heavier than alcohol, that leaving the tissue tends to remain at the bottom of the container. Thus the material to be dehydrated is always in the worst possible position. To avoid this, the author proposes a vessel, 35 or 40 mm. in diameter, with a widened base and a constriction in the middle which supports a non-corrosive wire gauze. The tissue is placed on the gauze with just enough alcohol in the vessel to cover its upper surface. This flask can be used in the same way for decalcification.—Jean E. Conn.

MURDOCK, S. E. A method for the removal of precipitate from tissues fixed in formaldehyde. *J. Tech. Methods*, **25**, 71-2. 1945.

A fine brownish-black crystalline pigment derived from laked blood frequently results from formaldehyde fixation. This may be overcome by the following

procedure: bring tissues to water; place in ammoniacal  $\text{H}_2\text{O}_2$  solution (25 ml. 3%  $\text{H}_2\text{O}_2$ ; 25 ml. acetone; 1 drop concentrated ammonia); let stand 1-2 hr. or until precipitate is removed; wash in water; stain as usual.—*C. Randall.*

### DYES AND THEIR BIOLOGICAL USES

DAVID, W. A. L. The quantity and distribution of spray collected by insects flying through insecticidal mists. *Ann. Appl. Biol.*, 33, 193-41. 1946.

In studying insecticides, it is helpful to measure the quantity of spray which flying insects accumulate from a mist dispersion. When the housefly (*Musca domestica* L.) and the yellow fever mosquito (*Aedes aegypti* L.) are placed in a chamber containing a mist of odorless kerosene dyed with Sudan III, droplets accumulate on both surfaces of the wings but not on any other part of the body. The dye is later found in the gut, feces, and malpighian tubules. To determine quantitatively the amount of an insecticidal mist which is accumulated, the spray is dyed with a known quantity of the dye, the insects are later extracted in odorless kerosene, and the extracts are compared with a set of prepared standards. From the amount of dye extracted, the quantity of the insecticide accumulated is calculated by assuming that with relatively nonvolatile materials the ratio of dye to insecticide does not undergo any appreciable change. This colorimetric method is used to study the effect on the quantity of spray accumulated, of changes in the properties of the mist dispersions and in the behavior of the insects. Within limits the dye accumulated increases with the activity of the insect and the size of the mist particles.—*H. P. Riley.*

GOMORI, G. Buffers in the range of pH 6.5 to 9.6. *Proc. Soc. Exp. Biol. & Med.*, 62, 33-4. 1946.

Three organic chemicals used separately with 0.1 N HCl were found to cover the range from pH 6.5 to 9.6. These substances are: (1) 2,4, 6-collidine (4-collidine) (Eastman Kodak Co.); (2) Tris (hydroxymethylaminomethane) and (3) 2-amino-2-methyl-1,3-propanediol (Commercial Solvents Corp.) They do not precipitate calcium salts and are inexpensive and stable. Values for pH and  $pK_b$  at 25° and 37° C. are given.—*H. A. Davenport.*

McBAIN, J. W., and GREEN, AGNES ANN. Solubilization of water-insoluble dye in soap solutions: effects of added salts. *J. Amer. Chem. Soc.*, 68, 1731-6. 1946.

The solubilization of orange OT (F.D. and C. orange No. 2) in aqueous solutions of soaps at 25° C. is described. In sufficiently dilute solutions of detergents, there is no solubilization, presumably because there are no colloidal particles in which the solubilized material can be incorporated. As suitable micelles form in the detergent solution upon increase of concentration, the amount of dye solubilized by each mole of soap rises to a constant value, which increases again only in the highest concentrations. Solubilization is not proportionate to the amount of hydrocarbon in the soap molecules or micelles, but rises rapidly with a slight increase in hydrocarbon content. In all concentrations, salts (KCl,  $\text{K}_2\text{SO}_4$ , KSCN, which by themselves are non-solubilizing) greatly increase the amount of solubilization by a given amount of soap.—*R. T. Whittenberger.*

MICHAELIS, L., and GRANICK, S. Metachromasy of basic dyestuffs. *J. Amer. Chem. Soc.*, 67, 1212-9. 1945.

Many basic dyestuffs are adsorbed by stainable substrates in two different shades of color, designated as the normal and the metachromatic color. As a model for a substrate stainable in the normal color, a 3% solution of nucleic acid at pH 4.6 is chosen; as a model for a substrate stainable in the metachromatic color, a solution of agar at pH 4.6 is chosen. All dyestuffs capable of metachromasy disobey Beer's law in aqueous solution, owing to the fact that with increasing concentration dimeric molecular aggregates of the dye molecules are formed. The absorption maximum of the dimer lies at shorter wave lengths than that of the monomer as it exists in dilute solution. In the presence of agar still higher molecular aggregates are formed and are absorbed by agar. It is probable that one cation of the dye is adsorbed primarily by an acid side chain

of the substrate and carries along an aggregate of other dyestuff molecules including their anions. The fact that metachromatic color in agar gives way, reversibly, to an approximately normal color at higher temperature corroborates the hypothesis of polymerization of the dye molecules. The absorption bands of these high polymers are displaced toward shorter wave lengths and are more diffuse than those of the monomer. The metachromatic effect is nearly independent of pH and cannot be attributed to formation of the free base. In contrast, in the presence of nucleic acid the absorption spectrum of all basic dyes is independent of the concentration of the dye and is similar to that of the dye in dilute solution. Beer's law is strictly obeyed. The peak of absorption is displaced slightly toward longer wave lengths by nucleic acid and the peak is lowered slightly. No polymerization takes place. Each cation of the dye is combined with one acidic side chain of nucleic acid to form a stoichiometrically well-defined salt-like compound. Little is known about the correlation of the chemical structure of a dye with its faculty of polymerization. Yet this faculty of polymerization is always correlated with the metachromatic effect.—*R. T. Whittenberger.*

OAKLEY, C. L., WARRACK, G. H., and VAN HEYNINGEN, W. E. The collagenase ( $\kappa$  toxin of *Cl. Welchii* type A). *J. Path. & Bact.*, **58**, 229-35. 1946.

The demonstration of collagenase is facilitated by diazotizing collagen to form a dye which, by going into solution, measures collagen digestion. The collagen-dye is prepared as follows: screen 1 lb. of hide powder (Baird & Tatlock) to 60 mesh; about 120 g. of screenings should be obtained. To a solution of 0.575 g. benzidine in 100 ml. of water containing 3 ml. concentrated HCl, cooled in an ice bath, add slowly 0.45 g.  $\text{NaNO}_2$  in 10 ml. of water. Allow to stand 10 min., then pour the tetrazotized benzidine into a chilled solution of 6.25 g. of crystalline sodium acetate in 500 ml. of water. To the mixture add with constant stirring 1.1 g. R-salt (Na salt of 2-naphthol-3,6-disulfonic acid) in 100 ml. of water followed by 20 ml.  $\text{N K}_2\text{CO}_3$ . A brick-red dye is formed. Meanwhile wash 80 g. 60-mesh hide powder by repeated suspension in and filtration from 10 liters of water and finally resuspend in 500 ml. water containing 30 ml.  $\text{2N K}_2\text{CO}_3$ . Add the dye to the suspension in 6 equal lots at 10 min. intervals. The color changes from brick-red to purple-red. After all dye has been added, add 25 ml.  $\text{2N K}_2\text{CO}_3$  and allow mixture to stand 10 min. Centrifuge or filter off the dyed hide powder and wash by resuspending 5 times in 5-liter quantities of water. Resuspend in 300 ml. of water, stirring constantly, and slowly add 1.7 liters of acetone. Repeat the acetone washings until the washings are no longer pink, filter off the dyed hide powder ("azocoll") and remove residual acetone at  $37^\circ\text{C}$ . Screen the azocoll to 60 mesh, dry in  $\text{P}_2\text{O}_5$  under reduced pressure and keep in 5-10 g. amounts in rubber-capped bottles under nitrogen.

Azocoll required as indicator should be suspended as needed in 1% Manucol IV (a polymer of *d*-mannuronic acid, Allbright & Wilson, Birmingham).—*S. H. Hutner.*

### ANIMAL MICROTECHNIC

CAMPBELL, BERRY, and NOVICK, ROSALIND. A quantitative method for the study of chromatolysis. *Proc. Soc. Exp. Biol. & Med.*, **61**, 425-7. 1946.

Six cell types are defined accurately and examples are given of their use in quantitative evaluation of cellular changes in the spinal cords of cats after root section. The sequence of changes is illustrated by charts and figures. The method differentiates chromatolytic changes which have gone on for varying periods of time.—*H. A. Davenport.*

DILLER, I. C. Smear methods for mammalian tissues, including tumors. *J. Tech. Methods*, **25**, 73-6. 1945.

Acetic orcein is very satisfactory for nuclear preparations of soft mammalian tissues, especially rapidly growing tumors. For tumor tissue, the method is as follows: tease bits of tissue into minute fragments with fine needles in a drop of acetic orcein (1 g. orcein dissolved in 45 ml. boiling glacial acetic acid; 55 ml. distilled water; cool and filter); press cover glass firmly down upon the prepara-

tion and complete dissociation of cells by tapping with a blunt instrument; store overnight in vapor chamber (95% alcohol); soak off cover glass in fluid alcohol; counterstain in fast green; rinse in two changes of 95% alcohol; mount in diaphane. Details in the cell body are sharper if the tissue is teased in physiological saline solution until it is semifluid. These preparations may be floated on any fixative. The disadvantage of the saline method is that fresh tissues must be prepared at once.—*C. Randall.*

GLEES, P., MEYER, A., and MEYER, M. Terminal degeneration in the frontal cortex of the rabbit following the interruption of afferent fibers. *J. Anat.*, 80, 101-7. 1946.

One of the authors (Glees) has developed an impregnation technic which he found very useful in the demonstration of the degenerating unmyelinated nerve fibers and terminals which were studied in this work. The technic consists in fixing a block of tissue for at least 5 days in 5% formal-saline, then cutting on the freezing microtome at 12-15  $\mu$ . The sections are washed well in distilled water and left for 24 hours in 50% alcohol with 5-10 drops of concentrated ammonia, re-washed in distilled water and placed in 10%  $\text{AgNO}_3$ , where they are left until brown (24 hours-5 days). Then, without washing, they are reduced in several changes of 4% formaldehyde (i.e. commercial formalin 1:10, made up with tap water). From this the sections are placed in the following ammoniacal silver solution: 10 ml. absolute alcohol; 10 ml. 20%  $\text{AgNO}_3$ ; ammonia added until the precipitate so formed is redissolved. Sometimes a few additional drops of ammonia seem to be needed to obtain the result desired. The sections remain in this solution for  $\frac{1}{2}$ -1 minute. The reduction in several changes of 4% formaldehyde is repeated; the sections are washed twice in distilled water, fixed in 5% hypo, washed well, dehydrated in graded alcohols, cleared in creosote, and mounted.—*Warren Andrew.*

HUSEBY, ROBERT A. Hydroxybenzene compounds as cytoplasmic fixatives. *Proc. Soc. Exp. Biol. & Med.*, 61, 122-5. 1946.

Mitochondria, secretion granules and other specific types of granulation are well preserved in tissues fixed in neutralized formalin-pyrogallol and formalin-resorcinol mixtures. Pyrogallol is added to 10% formalin in 3.5 to 7% concentrations; resorcinol in 4.5 to 9% additions. Both mixtures are alkalinized with 1% by volume of 1 N NaOH. Fixation time of 6 to 24 hr. is recommended. A discussion of staining granules in cytoplasm by several technics is given and illustrated by two figures. Hydroquinone and catechol mixtures are less effective than pyrogallol or resorcinol. This type of fixation is of special interest in connection with the centrifugal fractionation of cellular components and in tissue culture.—*H. A. Davenport.*

KELLER, G. J. A reliable Nissl method. *J. Tech. Methods*, 25, 77-8. 1945.

The following method overcomes the difficulty of demonstrating chromophilic substance of nerve cells in material in which fixation has been poorly controlled: bring sections to distilled water; 0.5% cresyl violet (Coleman and Bell or Grubler's), 3-5 min.; 2 changes of distilled water; 95% alcohol, 30 sec.; absolute alcohol, 30 sec.; xylene, 1 min.; equal parts of Canada balsam and xylene, 2 min.; absolute alcohol, 10-30 sec.; several changes of xylene. The last three steps may be repeated if differentiation is not complete. Carbol-xylene cannot be substituted for xylene.—*C. Randall.*

MACNEAL, W. J., and BLEVINS, A. Fixation of tissues by perfusion technique. *J. Tech. Methods*, 25, 54-60. 1945.

Fixation of tissues by perfusion technic is recommended as a supplementary method of preparing tissues for microscopic study. The authors prefer Helly's fluid ( $\text{K}_2\text{Cr}_2\text{O}_7$ , 2.5 g.;  $\text{Na}_2\text{SO}_4$ , 1 g.;  $\text{HgCl}_2$ , 5 g.; water, 100 ml.; 40% formaldehyde, 5 ml.) which causes prompt fixation.

A surgical specimen may be quickly weighed and measured, a portion removed for culture and one for fixation while in undistended state. The remainder of the specimen may be promptly perfused to remove blood and to fix the structures while the vascular spaces are distended. Finer details of structure may sometimes be more distinctly seen in tissues fixed by arterial perfusion.—*C. Randall.*

PAUL, PAULINE, and McLEAN, BETH BAILEY. Studies on veal. I. Effect of different internal temperatures on veal roasts from calves of three different weights. *Food Research*, 11, 107-15. 1946.

*Histological Studies:* Cooked muscles, 71° C. (160° F.), were sectioned on the freezing microtome at a thickness of 160  $\mu$ , and were stained with gentian violet to show the general distribution of connective tissue and to determine the arrangement of muscle fibers. Small pieces of raw muscle and of muscle cooked to 71 and 88° C. (160 and 190° F.) were fixed in formalin and Zenker's fixative. The Zenker-fixed pieces were embedded, sectioned, and stained with Mallory's triple connective tissue stain, while the formalin-fixed pieces were embedded, sectioned, and stained with Weigert's elastic connective tissue stain.

The changes in microscopic appearance brought about by cooking were (1) swelling of the collagenous fibers observed in the semitendinosus muscle, (2) partial breakdown of the connective tissue reticulum surrounding the muscle fibers, and (3) changes in the staining properties of the sections with the connective tissue of the cooked sample staining less intensively.—*William G. Walter*.

SALAZAR, A. L. *Résumé des travaux réalisés depuis 1941 jusqu'à 1945.* Centro de estudos microscópicos do Instituto para a Alta Cultura, Universidade de Porto. Porto, Portugal. 23 pp. 1946.

Salazar has summarized in French the work which has been done on cell structures and especially on connective tissue by the iron tannate method. Almost all of the papers are from his laboratory and were written in Portuguese so that such a summary is valuable to those who cannot read this language. A number of articles which have appeared in countries other than Portugal are included but the list is not complete. The bibliography will be found very useful to all users of the Salazar technic as well as to cytologists and animal histologists.—*B. F. Lutman*.

### PLANT MICROTECHNIC

GUILLIERMOND, A. La coloration vitale des chondriosomes. *Bull. d'Histol. Appl.*, 17, 225-37. 1940.

The author has studied in some detail the vital staining of the higher plants, Saprolegnia, and fungi, with particular reference to the vital staining of the chondriosomes, and has observed that dyes which stained chondriosomes in living cells when mounted on a slide would stain permanently nothing but the vacuoles in actively growing cells. The dye might stain the chondriosomes temporarily, but would soon be excreted into the vacuoles. The higher plants would grow normally with dye in the vacuoles, but the fungi would not grow until all the dye had been excreted from the plant. He concludes that the cells on the slide are in an abnormal condition and that staining of chondriosomes is not compatible with cellular growth.—*Jean E. Conn*.

### MICROORGANISMS

HENKE, R. J. Experiments on the differential staining method of Dold. *Zentralbl. Bakt., I Abt. Orig.*, 148, 132-46. 1941.

In Dold's method bacteria are stained with phenol-aniline green solution, then treated with a Lugol solution and decolorized in an alcoholic urea solution, which is followed by staining with Bismarck brown. When treated in this way some of the Gram-positive bacteria take the counterstain, others keep the original green color. The writer proves in a series of experiments that it is not the urea compound which is responsible for the action of the decolorizing agent. It is a special proportion between water and alcohol which causes this effect. So when smears with Gram-positive bacteria stained by a special coloring agent are treated with a solution of 88 vol. of alcohol and 12 vol. of water for 5 min., the Gram-positive group of bacteria can be differentiated into Dold-positive and Dold-negative forms. A certain group of bacteria is in a borderline position and minor changes in the technic may cause an irregular result.—*A. C. Buys*. (Courtesy *Biological Abstracts*).

JOHNSON, EDWIN A. An improved slide culture technic for the study and identification of pathogenic fungi. *J. Bact.*, 51, 689-94. 1946.

Sterile slide cultures of fungi on potato-dextrose agar formed good spores which adhered to the cover slip when it was removed carefully. These spore clusters were fixed by flaming, stained with 0.5% safranin (no specifications), and mounted in Clarite or balsam. Several species of fungi ordinarily difficult to identify were easily classified from such cultures.—*Virgene Kavanagh*.

KOPEL, M. A modification of the Giemsa stain for malarial parasites. *J. Tech. Methods*, 25, 61-2. 1945.

Absolute ethyl alcohol can be substituted for absolute methyl alcohol in the Giemsa stain for malaria parasites, both in fixing the smear, and in the stain itself. Nuclei stain red and cytoplasm blue. The results of the modification are adequate and dependable.—*C. Randall*.

LAMANNA, CARL. The nature of the acid-fast stain. *J. Bact.*, 52, 99-103. 1946.

Several experiments in staining bacteria with Ziehl-Neelsen's carbol fuchsin suggested to the author that the beading obtained resulted from the separation of the phenol and dye as a liquid phase. On this basis acid-fastness lies in the greater solubility of the phenol and dye in the cell constituents than in the decolorizing agent.—*Virgene Kavanagh*.

### HISTOCHEMISTRY

McCRONE, W., SMEDAL, ANNETTE, and GILPIN, V. Determination of 2,2-bis-p-chlorophenyl-1,1,1-trichloroethane in technical DDT. A microscopical method. *Ind. Eng. Chem., Anal. Ed.*, 18, 578-82. 1946.

The method depends on the influence of impurities on the rate of crystallization of the major component from the melt. It is applicable to a large number of important systems such as explosives, pharmaceuticals, dye intermediates, or almost any organic reaction product. As applied to the determination of p,p'-DDT in technical DDT, the method makes possible an analysis in 10 to 15 min. with an accuracy of  $\pm 0.5\%$ . A small sample is heated on a hot plate to a temp. not exceeding  $120^{\circ}\text{C}$ . The melt is mixed by stirring for about 1 min. One drop is placed on a slide, and a cover glass is pressed down firmly to give a thin film. If necessary, the sample is seeded at the edge of the cover glass with a crystal of p,p'-DDT to initiate growth. The sample is allowed to cool for 2 min. and a thermometer is placed on the microscope stage with the bulb near the sample. After 2 more min. the temperature is recorded and the crystal front focused with the eyepiece micrometer scale. The rate of growth of the p,p'-DDT is determined over a period of 5 to 10 min. The temperature is noted several times during the determination. The rate of growth expressed in microns in 5 min. and the average temperature are used in a standard graph to determine the per cent of p,p'-DDT.—*R. T. Whittenberger*.



# STAIN TECHNOLOGY

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## THE STAINABILITY OF NERVE FIBERS BY PROTARGOL WITH VARIOUS FIXATIVES AND STAINING TECHNIQS

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**ABSTRACT.**—The influence of the commonly used tissue fixing reagents, individually and in various combinations, on subsequent staining by protargol was studied. The reagents used were formalin, formamide, picric acid, acetic acid, paranitrophenol, pyridine and chloral hydrate. Paraffin sections from intestine and peripheral nerve of cat, dog, monkey and rat were stained with protargol after fixation in various experimental mixtures of the fixing reagents. Satisfactory nerve stains of intestine were not obtained with regularity after any one fixing and staining procedure. Good fixation and staining appeared to be influenced by properties inherent in the tissue itself and showed marked variations from animal to animal even in the same species. Stains of nerve fibers in peripheral nerve trunks were much more easily obtained than in the intestine where good stains were sporadic and unpredictable. The use of a mixture of 0.5% protargol and 0.1% fast green FCF, is proposed as a silver-dye staining medium.

This work is an extension of a type of investigation reported previously (Bank and Davenport, 1940) and consists of a series of experiments in which a number of kinds of fixing fluids were used and the tissues subjected to several variations of staining on the slide with protargol. Intestine and peripheral nerve trunks were used in the present series and the animals represented were cat, dog, monkey and rat. The expectation was that a procedure suitable for staining autonomic nerve fibers in the intestine could be found. This expectation was realized only partially and it appears that the use of protargol for such staining will not immediately supplant the customary use of supravital methylene blue.

Hill's (1927) figures of the intestinal plexuses were used for reference. The various components which she illustrated were identified with the exception of nerve fibers in the villi. Not all compo-

<sup>1</sup>Contribution No. 466 from the Anatomical Laboratory of Northwestern University Medical School, Chicago Ill.

nents stained in the same specimen, and about half of our preparations failed to give nerve staining except in the mesentery. Fibers in the mucosa were seen occasionally and then only at the bases of the villi or just inside of the muscularis mucosae. Fibers and cells of the submucous plexus, even though stained, were often made hard to recognize by the overstaining of the muscularis mucosae and associated connective tissue. A favorable section sometimes showed the bands of fibers passing through the circular layer of

TABLE 1. COMPOSITION OF FIXING FLUIDS

No.	Per cent concentration in 40% ethyl alcohol							
	Form- alin	Form- amide	Picric acid, sat. aq.	Acetic acid, glac.	p-nitro phenol	Pyri- dine	Chloral hydrate	No. of times used
1	10	—	—	—	—	—	—	7
2	10	5	—	—	—	—	—	6
3	10	5	50	—	—	—	—	6
4	10	—	—	5	—	—	—	4
5	10	—	50	5	—	—	—	11
6	10	—	—	5	5	—	—	3
7	10	5	—	—	5	—	—	2
8	10	—	—	—	—	5	—	1
9	10	—	50	—	—	—	—	2
10	10	—	50	—	—	5	—	1
11	—	10	—	—	—	—	—	4
12	—	10	—	—	—	—	10	3
13	—	10	—	—	5	—	10	3
14	—	—	—	—	—	—	10	1
15	—	—	60	—	—	—	—	4
16	—	10	60	—	—	—	—	6
17	—	10	60	—	—	5	—	2
18	—	10	—	—	5	5	—	7
19	—	10	—	5	5	—	—	1
20	—	—	—	—	5	—	—	2
21	—	10	—	—	5	—	—	7
22	—	10	—	—	—	5	—	1
23	—	10	50	5	—	—	—	5
24	—	—	55	5	—	—	—	5
25	—	—	55	5	—	—	10	1
26	—	—	—	5	5	—	10	5
27	—	10	—	5	5	—	10	4
28	—	—	—	—	5	—	10	4

muscle (Fig. 1, 3, and 5). Sharp and well differentiated staining of the myenteric plexus was the exception rather than the rule. It should be noted (Fig. 2 and 6) that many of the nerve fibers (or fibrils?) are extremely small and some stains which looked poor under low power were seen to be good under oil immersion.

#### METHODS

*Fixation.* The following reagents were used: formalin, formamide, picric acid, acetic acid, paranitrophenol, pyridine, chloral hydrate,

ethyl alcohol, and distilled water. After making a preliminary test with alcohol concentrations of 30, 50 and 70% as the vehicle for the other fixing agents, it was decided that 40% would probably give as good results with staining as higher concentrations and less shrinkage, hence the experimental fixing fluids contained approximately 40% alcohol. Table 1 lists the mixtures tried. Specimens were fixed for 24 hours and dehydrated, without washing in water, through 70 and 95% alcohol, two changes of absolute alcohol, then xylene and embedded in paraffin.

*Material.* Pieces of small intestine from anesthetized or decerebrate animals were removed either during life or immediately after death. Pieces of sciatic or brachial nerves were fixed in the same bottles with the intestine and both types of tissue taken together through subsequent processes. This permitted a comparison of the staining results obtained on fine and coarse nerve fibers in both types of tissue.

Eight different fixing fluids were used as a rule on the specimens from one animal. The total number of times a given fluid was used is shown in the table, and since most of them were used on a number of different animals, it was possible to see whether any peculiarity of staining results was due to fixation or to the source of the material.

*Staining.* Five routines with protargol as the staining agent were used.

1) Bodian's (1936) stain: 1% protargol plus 5 g. (per 50 ml.) of clean, bright granulated copper.

2) Two-hour method (Davenport, McArthur and Bruesch, 1939, modified): 5% aqueous  $\text{AgNO}_3$  for 1 hour at 60°C. followed by 0.2% protargol for 1 hour at room temperature.

3) Plain protargol, 0.5%, for 24-48 hours at 37° C.

4) Protargol, 0.5%, plus 0.1% aniline blue (CC, No. NK 6) for 24-48 hours at 37°C.

5) Protargol, 0.5%, plus 0.1% fast green FCF (CC, No. NGf 6) for 24-48 hours at 37° C.

The last two staining baths were used to determine whether the affinity of the acidic dyes for connective tissue would block it against staining with protargol and thereby give more selective nerve fiber stains. Foley (1943) has used fast green as a counterstain but we have been unable to find any reports of its being mixed with protargol. Controlled observations were made by checking the results of procedures 4 and 5 with procedure 3.

Treatment of the slides after protargol was not varied and consisted of reduction, toning with gold chloride, oxalic acid (second reduction),

hypo, dehydration and covering— with appropriate washing in water where required. The formula of the reducing solution used with all stains is given in the appendix of this article. This was the usual reducing solution with Kodalk added to insure sufficient alkalinity for maximum reduction of the protargol.

## RESULTS

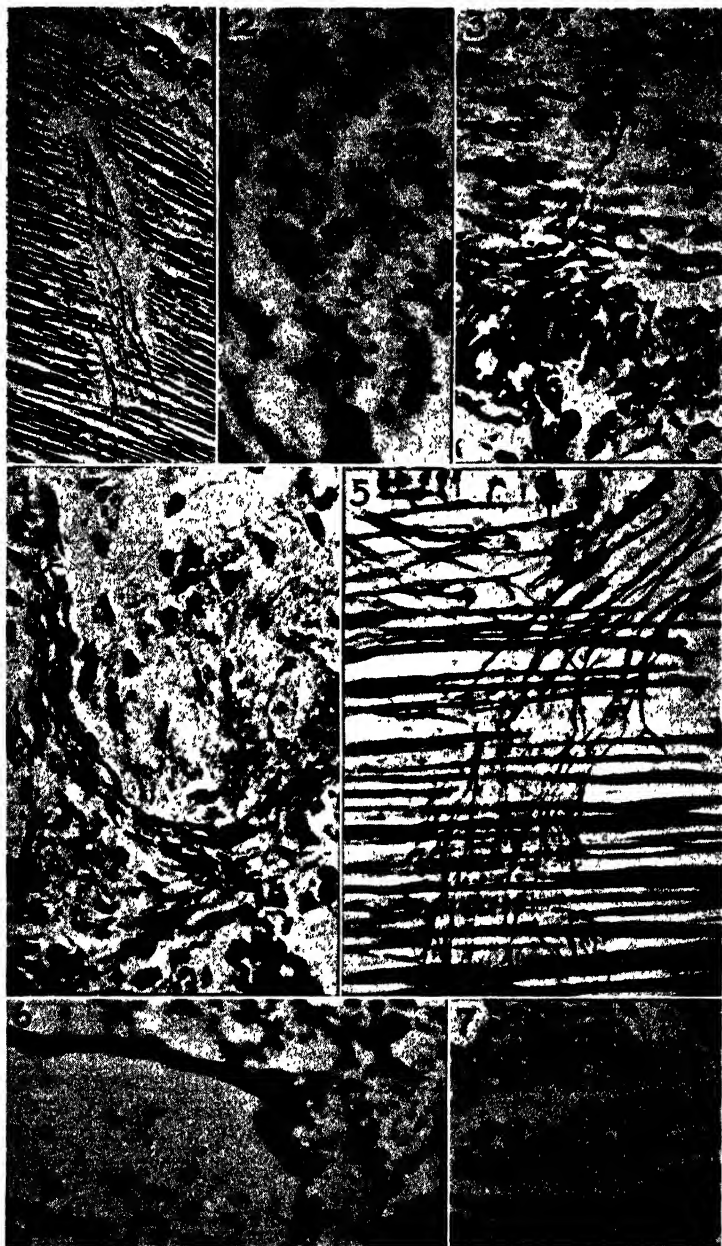
1. *Intestine.* The outstanding feature was the effect of formalin as a component in a fixative. It uniformly gave better fixation but reduced the affinity of the tissue for protargol. The larger nerve fibers sometimes stained sharply but the smaller ones failed to stain. Connective tissue and smooth muscle usually remained pale. Addition of picric acid to formalin enhanced the stainability of smaller nerve fibers and also that of other tissue components, hence the entire section was darker without any advantage in the differentiation of neural elements. Of all solutions used, formalin-picric-acetic (Bouin's) gave the best tissue preservation whether in aqueous solution or Bouin's mixture in 40% alcohol. Staining of the myenteric plexus was occasionally rather good (Fig. 2).

Picric acid used either alone or in combination was an effective fixing agent. It was removed in a routine way before staining with protargol by treating the slides with 1% ammoniated 95% alcohol in the xylene to water series. Material so fixed usually stained readily, but nerve fibers were often stained less well than connective and other tissue elements.

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Plate 1. Photomicrographs of 10 $\mu$  sections of intestine which illustrate the type of staining obtained with protargol.

- Fig. 1. Dog. Fascicle within the circular layer of muscle. Protargol-fast-green stain. Fixative 18 (Table 1).  $\times 500$ .
- Fig. 2. Monkey. Cross section of intermuscular fascicle of myenteric plexus. Protargol-fast-green. Fixative 5.  $\times 1000$ .
- Fig. 3. Rat. Fascicle which connects the myenteric and submucous plexuses. Plain protargol stain. Fixative 16.  $\times 500$ .
- Fig. 4. Cat. A fascicle in the submucous plexus which is in close relationship with the glandular tissue. Bodian stain. Fixative 28.  $\times 500$ .
- Fig. 5. Cat. Plexiform fiber arrangement in the circular and deeper portion of the longitudinal muscular layers. Protargol-fast-green stain. Fixative 28.  $\times 500$ .
- Fig. 6. Cat. Fascicle in the longitudinal muscle layer which joins a transversely sectioned bundle lying between the two muscular coats. Silver-nitrate-protargol 2-hr. stain. Fixative 9.  $\times 1000$ .
- Fig. 7. Cat. Intermuscular plexus with longitudinal and cross sections of nerve fascicles. Protargol-aniline-blue stain. Fixative 23.  $\times 500$ .



Acetic acid was not used alone. In combination with other ingredients it appeared to reduce the affinity of connective tissue for silver by reason of the characteristic swelling of fibrillar elements. No definite advantage in its use appeared except in Bouin's mixture, since formalin-picric mixtures without acetic gave poor results.

Paranitrophenol was used because it is one of the essential ingredients in Petrunkevitch's fixing fluid (1933). Its chemical relationship to picric acid (2-4-6 trinitrophenol) adds to the interest of its use in fixation of tissue. Observations indicate that its action as a protein coagulant was weak, if present at all, and that it did not influence staining. It appears to have an anti-enzymatic action however. Intestinal villi of specimens placed in 40% alcohol alone for 24 hours showed considerable tissue autolysis whereas the villi of similar tissue in 40% alcohol with 5% *p*-nitrophenol added were not autolyzed. It may be considered as a preservative rather than an active fixing agent.

Pyridine combined with *p*-nitrophenol and formamide gave some preparations which had the best differentiated nerve fibers (Fig. 1), but fixation was very bad. Pyridine always contributed to the shrinkage and this was particularly noticeable in combination with formalin. An ideal combination (not attained in this series of experiments) would be fixation as good as that seen with Bouin's mixture with the staining effects seen after fixatives containing pyridine.

Chloral hydrate alone or with formamide gave much shrinkage of smooth muscle fibers and other cytoplasmic tissue elements. Combined with acetic acid and picric acid, the fixation picture was better but differential staining of nerve fibers was poorer. Combined with acetic acid and *p*-nitrophenol the amount of shrinkage was less and differential staining of nerve fibers and ganglion cells rather good. The staining of blood capillaries was a prominent feature not seen in comparable tissue fixed in Bouin's fluid.

2. *Peripheral nerve trunks.* The chief faults seen in the fixation of peripheral nerve trunks are excessive shrinkage, cracking (with resultant mosaic-like cross section), and clumping of the fine unmyelinated nerve fibers. After certain types of fixation the unmyelinated fibers subsequently fail to stain even though the coarse ones stain very well. In these experiments the observations of Holmes (1943) that plain formalin is not a good fixative, while picric acid is very good, have been fully confirmed.

The relative merits of the different reagents used were not particularly striking, hence a brief general discussion will cover the findings.

Whereas intestine deteriorates badly in 40% alcohol alone, nerve trunks may yield an acceptably stained preparation after "fixation" for 24 hours in that fluid. This difference seems attributable to the absence of any very active tissue enzymes, which cause autolysis, in the nerve.

Like the intestines, nerve trunks showed much variation from animal to animal in fixation and staining reactions. This variation was not particularly related to species. There were "good" cats and "bad" dogs and vice versa. In a bad animal poor fixation, poor staining, or both, were outstanding in all fixations and all staining technics. In the good animal the reverse was true. Rat tissue was somewhat more refractory than that of the larger mammals. This peculiarity of good and poor staining has probably been observed in most technical laboratories, but it has not been given much emphasis in the literature. The present series of experiments has provided a rather convincing demonstration of fixing and staining variations referable solely to the source of the tissue.

A few comments on the individual reagents used seem to be merited. The amount of alcohol in the fixing mixture from 0 to 50% is not critical. Higher concentrations lead to greater shrinkage which do not necessarily enhance staining. Formalin gives a minimum of shrinkage of myelinated fibers but unmyelinated fibers often remain unstained or are not discrete. Formamide causes a moderate amount of shrinkage of large fibers and appears to enhance stainability of unmyelinated fibers. Picric acid either alone or combined with acetic acid or formamide is a reliable and usually satisfactory fixing agent. Bouin's fluid (with or without alcohol) is suitable for nerve trunks if relatively unshrunk myelinated fibers are desired. The staining of unmyelinated fibers is less dependable after it than after fixation in picric-acetic without the formalin. Acetic acid in some cases seems to have no effect on the subsequent stainability of fine fibers but lessens it in others. Advantages of its use are still questionable. Paranitrophenol is a reagent which leaves little evidence of its effect on fixation or staining. Nerves fixed in its solution alone or combined with formamide give a fixation picture much like that obtained from plain alcohol but with better tissue preservation. Pyridine enhances staining of fine fibers, also shrinkage, but fascicles of unmyelinated fibers remain unclumped. Chloral hydrate is likely to cause considerable shrinkage and distortion. This appears to be made worse rather than better by combining it with acetic acid, formamide and *p*-nitrophenol. Nerve fibers are usually readily stainable after its use, however.

3. *Staining procedures.* When a certain animal yielded tissue in which the nerve fibers were readily stainable by one staining technic they usually stained with all technics. The best differentiated stains were obtained by Bodian's method. Since the action of the metallic copper is suppression of staining of connective tissue, and since staining conditions which favor the finer nerve fibers are similar to those for connective tissue, the smaller fibers were sometimes differentiated out also. Staining sometimes failed to occur by this technic when protargol alone was used successfully. Conversely, tissue which tended to overstain with protargol alone was likely to give better results with copper-protargol.

The two-hour method gave less satisfactory results on intestine than peripheral nerve. Poor differentiation of nervous elements with strong staining of glandular and muscular tissue was the tendency. A few moderately good stains were obtained after Bouin's fluid, of which Fig. 2 is an example.

After studying more than 1000 slides, we must admit that use of protargol alone in 0.5% concentration and staining for 24-48 hours at 37°C. is about as reliable a silver stain as more complicated modifications. It lacks the brilliant differentiation of copper-protargol (Fig. 4) but may succeed when both copper-protargol and the 2-hour procedures fail. (Fig. 3)

Mixing a dye with protargol offers a theoretical possibility of connective tissue suppression. The following dyes were tried: hematoxylin, gallocyanin, phloxine B, orange G, fast green FCF, and aniline blue W. S. These were added to 0.5% protargol to make a final dye concentration of 0.05% in some experiments and 0.1% in others. With hematoxylin and gallocyanin, nerve fibers did not stain, but a rather pale diffuse staining of most other tissue elements occurred. The phloxine B and orange G did not inhibit nerve fiber staining but showed no advantage over plain protargol. The fast green and aniline blue apparently effected improvement in the differential staining. (Figs. 1, 2, 5 and 7.) Of the two, we prefer fast green, since it comes through the processing better than aniline blue. Acidification of the final alcohol series (used preceding xylene and covering) helped to retain the color. Five drops of glacial acetic acid in each 50 ml. of alcohol was adequate acidification.

#### COMMENTS

Unless some newer and better routines for fixation and staining of intestine can be devised, the on-the-slide procedures with protargol are likely to give a liberal percentage of disappointments. If one were able to prepare a large series from many animals and select

(1) Best concentration of basic fuchsin solution. In most, if not all cases 0.5 g. of dye per 100 ml. of water is used (Carlson, 1936; Coleman, 1938; DeTomasì, 1936; McClung, 1937; Rafalko, 1946; Stowell, 1945). Shriner and Fuson (1940) indicate 0.1 g. of dye per 100 ml. of water in preparing the Schiff reagent. I have obtained satisfactory preparations with both concentrations.

(2) Best concentration of sulfurous acid. I have made no comparison as to the preference of adding a saturated solution of sulfurous acid to the basic fuchsin solution, in equal proportions (present method), or saturating the basic fuchsin solution directly with sulfur dioxide (Rafalko's recently described method). In the former case, the staining solution would of course be no more than half saturated with sulfur dioxide.

(3) Best concentration of HCl in the fuchsin-sulfurous acid solution. DeTomasì (1936) recommended addition of HCl to the staining solution when American fuchsin are used. Whenever the chloride salt of the dye (pararosaniline hydrochloride) is used, some HCl will of course be produced through the action of sulfurous acid on the dye. Whether this quantity is sufficient when the present method is used, or whether more should be added, remains yet to be determined.

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## MALT DIASTASE AND PTYALIN IN PLACE OF SALIVA IN THE IDENTIFICATION OF GLYCOGEN<sup>1</sup>

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**ABSTRACT.**—Digestion in 1% U.S.P. malt diastase or in 1% ptyalin at 37°C. for 1 hour is an effective substitute for the salivary digestion test used by Bauer, by Bensley and others for the identification of glycogen. Actually the test is not specific for glycogen, since diastase, ptyalin and amylopsin digest other polysaccharides than glycogen, notably starch. In animal tissues this should produce no confusion. Of the two samples tested, the malt diastase proved somewhat more effective than ptyalin and can be fully recommended for sharpness of results.

The enzymes should be dissolved in a buffered neutral saline solution consisting of 8 g. NaCl, 1.3 g. Na<sub>2</sub>HPO<sub>4</sub> and 0.8 g. NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O in 1 liter of water. This solvent by itself does not remove glycogen from liver sections in 1 hour at 37°C.

Enzyme tests should be done on uncollodionized sections. Since collodionization permits demonstration of larger amounts of glycogen by both the Best and Bauer methods, this step should be interposed after the digestion test and before the specific glycogen stain.

In recent work on glycogen and other polysaccharides in tissue we have had occasion to make extensive use of enzyme digestion tests for the further identification of the polysaccharide in question. At first we used C. Bensley's (1) salivary digestion technic, but this proved to be inconvenient on account of the quantities of human saliva required. On this account we were led to try prepared enzymes.

At first we were using collodion-covered sections for digestion tests, since we had found that these were superior to uncoated paraffin sections for the Best and Bauer glycogen technics. It soon became evident that results were irregular and somewhat unreliable. Liver glycogen was found to resist salivary and enzyme digestion in irregular areas.

The question arose next, if the collodionization were omitted would not any demonstrated loss of glycogen be due simply to solution and not to enzyme action, and hence the test be of no value in specific identification of the removed material. Hence the following

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experiment was devised (Table 1). Seven sets of 5 paraffin sections each of livers previously shown to contain considerable quantities of glycogen were prepared. The first set was the positive control. These sections were deparaffinized with xylene, carried into 100% alcohol and then immersed in 1% collodion in a mixture of 50:50 ether and 100% alcohol for 5 minutes, drained on edge for 1 minute and hardened at least 5 minutes in 80% alcohol. Next, sections were washed in tap water and treated 1 hour in 5% chromic anhydride ( $\text{CrO}_3$ ) solution in water, pouring the solution off and back in again every 10 minutes to insure evenness of action. Then sections were washed 5 minutes in running water and immersed for 15 minutes in Schiff reagent, with periodic agitation; in 3 successive

TABLE 1. EFFECTS OF PRE- AND POST-COLLODIONIZATION ON PTYALIN DIGESTION OF GLYCOGEN, WITH SALINE CONTROLS

Test No.	Treatment				Results
	Pre-collodionized	Digested 1 hr. in	Post-collodionized	Stained by Bauer	Amount Glycogen remaining
1	+	-	-	+	+++
2	+	neutral saline	-	+	+++
3	+	1% ptyalin	-	+	± to +++
4	-	neutral saline	-	+	+++
5	-	1% ptyalin	-	+	± to -
6	-	neutral saline	+	+	+++
7	-	1% ptyalin	+	+	± to -

The above table gives the essential steps of the technics employed in proper sequence. For the full details consult the text.

90-second baths of M/20 sodium bisulfite ( $\text{NaHSO}_3$ ); and finally in running water for 10 minutes. Next they were counterstained 2 minutes in the Lillie Mayer acid hemalum (2), colored blue in tap water containing 2 or 3 drops of saturated sodium carbonate solution in 500 ml., dehydrated, cleared and mounted by an alcohol, alcohol and xylene, xylene, clarite sequence.

The Schiff reagent used in these tests was prepared as follows: Dissolve 1 g. basic fuchsin (C. I. No. 677) in 100 ml. boiling hot distilled water. Filter while hot and add 2 g. sodium bisulfite ( $\text{NaHSO}_3$ ) and 20 ml. N/1 hydrochloric acid. Stopper and let stand overnight at room temperature in the dark. (The solution is

now slightly brownish yellow.) Add 300 mg. finely ground charcoal, shake 1 minute and filter. The solution should now be a clear pale yellow. It should be stored at 0° to 5°C. and when so stored can be used for some months at least. It should not be returned to the stock bottle after use, and should be discarded when it turns pink.

We repeat, the *first* set of sections of glycogen-containing livers were stained by the above Bauer technic after collodionization. The *second* set were collodionized and hydrated as above, then immersed 1 hour at 37°C. in the enzyme solvent, consisting of 8 g. sodium chloride (NaCl), 1.3 g. anhydrous disodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) and 0.8 g. acid sodium phosphate monohydrate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ) in 1 liter of distilled water. Sections were then stained as before by the Bauer technic. The *third* set were treated as the second, except that 1% ptyalin in the above enzyme solvent was used after collodionization. The *fourth* and *fifth* sets were not collodionized at any stage. In the fourth the Bauer reaction was preceded by an hour's immersion in the enzyme solvent at 37°C., in the fifth set in 1% ptyalin in the same solvent for 1 hour at 37°C. The *sixth* and *seventh* sets were deparaffinized, hydrated and immersed respectively in the buffered saline and in 1% ptyalin as before for 1 hour at 37°C. They were then dehydrated with alcohols, soaked 5 minutes in 1% collodion, drained, hardened, hydrated and stained as in the first or control series.

The control series and the three series soaked an hour in buffered neutral saline all showed the same considerable quantities of glycogen in the livers. In the series digested with ptyalin after collodion treatment a moderate quantity of demonstrable glycogen remained near the surfaces of the blocks, less in the centers. In two of the livers the reduction of glycogen as compared with the controls was quite marked, in one it was moderate and in two scarcely or not at all perceptible. In the two series in which ptyalin digestion was performed either before or entirely without collodionization, glycogen was completely removed from three livers, in two of which small patches of glycogen-containing cells were seen in the subcapsular zone only.

On a preliminary series, a sample of amylopsin (Eimer & Amend 1-28) and one of ptyalin (Eimer & Amend 1-28) had been found to behave similarly on collodionized sections, so further testing was done only with the ptyalin. A sample of malt diastase U.S.P. IX (Eimer & Amend Y674) had failed to remove glycogen from collodionized sections, so this was tested further in the hope that we might have a means of distinguishing glycogen from starch.

For further testing 1% ptyalin and 1% diastase in the foregoing buffered saline solution were used on a series of livers in which glycogen was demonstrable by the collodion Bauer technic. Collodionization after enzyme digestion was practiced throughout because this procedure had been found to demonstrate more glycogen than the Bauer reaction without prior collodionization when enzyme digestion was not practiced.

Glycogen deposits in four hearts were completely removed by both enzymes. Glycogen in skeletal muscle was completely removed by both enzymes in two sections, completely by diastase and partially by ptyalin in one, traces remained after diastase and larger amounts after ptyalin in two, traces after diastase and none after ptyalin in one. Glycogen was completely digested by both enzymes in one example of renal pelvic epithelium, one of basal cells of colonic glands, three of bronchial smooth muscle, two of cartilage cells, one of hepatic artery muscle, and one of pulmonary arterial muscle. Traces remained after ptyalin but not after diastase in one example of bronchial muscle and one of hepatic arterial muscle. Livers showed complete removal after both enzymes in 50 instances, complete digestion with diastase and traces to even moderate amounts remaining after ptyalin in 47, traces after diastase and somewhat more after ptyalin in four, a considerable amount after both in one. The last probably represent slides improperly exposed to the enzyme action.

The staining of mucus and of cartilage matrix was not affected by diastase or ptyalin digestion.

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## PARAFFIN SECTIONS OF TISSUE FRAGMENTS

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Pathologists are frequently called upon to examine minute fragments of tissue obtained by aspiration or other similar means. Although the smear technic is generally satisfactory for this purpose, there are occasions when sections are preferable. This is the case when study of the interrelationship of cells is desired, or when one wishes to avoid the distortion produced in cells by smearing.

Many methods have been devised to prevent loss of tissue fragments when carrying them through the paraffin technic. No claim for much originality is made for the method to be described. It does, however, prevent all loss of tissue; indeed, by this method it is possible to obtain paraffin sections of the free cells of tissue fluids.

The fragments are retained in a single test tube during the various steps of procedure. It has been found that a tube one centimeter in diameter and six to eight centimeters in length is suitable for most specimens. In the case of fluids containing free cells, some fluid is placed in the tube and the cells are thrown down by a centrifuge. After removal of the supernatant with a Pasteur pipette, the desired fixative is added to the tissues in the tube.

After fixation, the tube is spun if necessary to secure packing and the supernatant fixative removed by pipetting. By this use of the centrifuge and pipette after treatment with each reagent, the tissues in the tube are subjected in turn to the chosen dehydrating and clearing reagents. It is desirable to keep the tube corked during these steps.

When the tissues are ready for the paraffin bath, the tube is again centrifuged, the supernatant removed and molten paraffin added. The tube, uncorked, is quickly placed in the oven.

At, or near the end of paraffin impregnation, a tight cork is replaced in the tube. By setting the tube in the inverted vertical position in the oven, the fragments are allowed to settle down on top of the cork.

The paraffin is then allowed to solidify with the tube still maintained in the inverted position. After removal of the cork, the bottom of the tube is snapped or broken off. The pencil of paraffin is pushed out, and the portion containing the tissues cut off and sealed

to the chuck of the microtome. The circular shape of the block is not a disadvantage in sectioning.

The method can be modified according to individual requirements. For instance, some may prefer to use a piece of glass tubing with a cork at one end rather than a test tube.

## BISMARCK BROWN AS A STAIN FOR MUCOPROTEINS

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**ABSTRACT.**—Bismarck brown has been used for many years as a stain for mucin. But some types of mucoprotein are so water-labile that they cannot be demonstrated by the aqueous or weak alcoholic solutions usually employed. It has been found that Bismarck brown in slightly acidified, strong alcoholic, solution stains mucin. A simple method is given for using this solution for staining water-stable mucoproteins. Another method is included in which full precautions are given for avoidance of water at all stages subsequent to fixation. This method must be used for the more water-labile mucoproteins. By the use of these methods, it has been possible to demonstrate a wide range of mucoproteins including those of the mast cells of Hardie and that of the zona pellucida of the graafian follicle.

Bismarck brown (C.I.331), also known as vesuvin or phenylene brown, was one of the first azo dyes to be prepared. It is formed by a combination of two molecules of *m*-phenylenediamine with one molecule of tetrazo-*m*-phenylenediamine. It was used by Weigert (1878) as a nuclear stain. List (1885) employed it in aqueous or absolute alcohol solution for staining mucin; nuclear counterstaining was obtained with methyl green. Mann (1902) advised a solution in 25% methyl alcohol to minimize the formation of precipitates; he counterstained with hematoxylin. Conn (1940) modified the method of List slightly.

Only by using strong alcoholic staining solutions were Hardie and Wesbrook (1895) able to retain the very water-labile mucoproteins in the mast cells of the villi of the intestine. List (1885), Langeley (1901) and others in the past emphasized the great lability of mucoproteins to water even after fixation. This point has more recently been observed again (Leach, 1938; Carleton and Leach, 1939). So it seemed desirable to use Bismarck brown in strong alcoholic solution as did List, rather than in the aqueous solutions more recently recommended. I have found that not only is this possible but it leads to more specific staining. Addition of hydrochloric acid, aluminum chloride or ferric chloride still further increases specificity. Mann (1902) pointed out that it is essentially the addition of alcohol and aluminum chloride which converts hematoxylin and carmine from nuclear to mucin stains.

An alcoholic nuclear stain must be employed when a water labile mucoprotein is to be demonstrated. A modified Weigert's hematoxylin (Weigert, 1904) gave a suitable color contrast. It gave the best results when ferric chloride had been used in the Bismarck brown solution.

Mucoproteins vary in their lability to water. Some are quite resistant and may be stained by Bismarck brown without any special precautions (Method A.). Others show extreme lability and can only be demonstrated satisfactorily by observing all the precautions advised in Method B.

*Method A.* For water stable mucoproteins:—

- 1) Fix, dehydrate, clear, section and mount by any desired technic.
- 2) Take sections down to water, using iodine and hypo if necessary.
- 3) Rinse in acid alcohol—2 minutes.
- 4) Stain in Bismarck brown solution— $\frac{1}{4}$  to 12 hours.

Dissolve 1.5 g. of Bismarck brown in 100 ml. of 70% alcohol containing 0.5 g. of ferric chloride. The solution should be filtered after standing overnight. Filtration from time to time is necessary.

- 5) Rinse in 70% alcohol.
- 6) Counterstain nuclei with hematoxylin—preferably Weigert's hematoxylin (Weigert, 1904).
- 7) Place in tap water until blue.
- 8) Dehydrate, clear and mount.

Results: Many types of mucoprotein—brown.

Nuclei—blue-black.

*Method B.* For water-labile mucoproteins:—

- 1) Fix in Zenker-formol (Helly's fluid).
- 2) Transfer to 90% alcohol with iodine added.
- 3) Dehydrate, clear, post-fix in alcohol (Leach, 1945); clear again and embed, preferably by the methyl-benzoate-celloidin method.
- 4) Section, but avoid cooling the cut face of the block in water as is usually done to facilitate section cutting.
- 5) Flatten out the sections on 70% alcohol or 50% diacetin (Carleton and Leach, 1939).
- 6) Take sections down to 70% alcohol.
- 7) Treat with iodine in 70% alcohol.
- 8) Remove excess iodine with 70% alcohol.
- 9) Acid alcohol, 2 minutes.
- 10) Bismarck brown solution (see above), 15 minutes to 12 hours.

- 11) Rinse in 90% alcohol.
- 12) Stain in the following modified iron hematoxylin of Weigert (1904) for 10 minutes:
  - A. Hematoxylin 1% in absolute alcohol.
  - B. Distilled water, 100 ml.  
Hydrochloric acid, 1 ml.  
Ferric chloride, 30%, 4 ml.Mix 1 volume of A with 1 volume of B. Add 2 volumes of absolute alcohol.
- 13) Rinse in 90% alcohol, then in 96% alcohol.
- 14) Absolute alcohol containing 0.1% potassium acetate (Mayer), 2 minutes.
- 15) Rinse in absolute alcohol.
- 16) Dehydrate, clear and mount.

Results: All types of mucoprotein—brown.

Nuclei—blue-black:

Observation is facilitated by use of a green light filter.

Some of the precautions here advised to avoid contact with water may often be omitted; but there are so many variables to consider that it is impossible to give specific advice on this point for any given type of mucoprotein.

Using this method it has been possible to demonstrate all the usual types of mucoprotein such as that of goblet cells, salivary and oesophageal gland cells, epithelial cells of the cervix of the uterus and mast cells. In addition, mucoprotein in the surface mucoid, neck mucoid and cardiac cells of the stomach and in Brunner's glands is well demonstrated. The mast cells of Hardie and Wesbrook (1895) which occur in the connective tissue of the villi of the intestine can be shown as clearly as in the original excellent but neglected drawings.

Faintly stainable material has been found in the cells of the convoluted tubules of the kidney corresponding in position to the alkaline phosphatase as demonstrated by the Gomori (1939) technic. This would seem to indicate that phosphatase is of the nature of a mucoprotein. It is of the labile type and dissolves and diffuses readily in water. A similar lability of phosphatase was mentioned by Gomori (1939) who advised celloidin coating of the section to minimize diffusion. In view of these facts it seems quite unwarranted to accept the cytological localization of phosphatase until it can be satisfactorily demonstrated by use of an alcoholic staining method. This would be possible by further improvement of a mucoprotein stain such as this Bismarck brown method.

The follicular fluid and the zona pellucida of the ovarian follicle are stained. This is in accordance with the view of Maclean and Rowlands (1942) and Rowlands (1944) that the ovum is surrounded by a mucoprotein.

Connective tissue mucoid in cartilage, cornea and umbilical cord are also stained.

There are three disadvantages of the method.

1) Slow fading occurs. Preparations made 2 years ago are still usable though definitely faded.

2) Staining of elastic fibers sometimes occurs. This may be observed too with other mucoprotein stains.

3) The granules of serous cells sometimes stain. This can often be minimized by adjustment of the strength of the ferric chloride added to the Bismarck brown solution and by adjustment of the staining time.

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## LABORATORY HINTS FROM THE LITERATURE

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A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOK AND PAPERS FROM OTHER JOURNALS  
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

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### MICROSCOPE AND OTHER APPARATUS

**SMITH, BURGESS.** Method for making filters transmitting the near ultra-violet and absorbing visual light. *Science*, 104, 490-1. 1946.

Filters are prepared by the following method: Dissolve 0.4 g. methylene blue (Heller and Merz Blue 2 B Dustless) in 100 ml. of hot water containing 1 drop of glacial acetic acid. Make a similar solution of Fuchsin RTN Powder and Phosphine 3G, 100%, omitting the acetic acid. Dyes of other manufacture may be used, the differences in absorption being compensated by altering the concentrations. Make a 10% solution of gelatin by allowing it to stand until it is swollen and dissolve by gentle heat. Add 2 drops of glycerin to each 25 ml. Pipette 10 ml. of the phosphine, 8 ml. of the methylene blue and 3 ml. of the fuchsin into a cylinder, and add 19 ml. of the gelatin. Mix thoroughly and, if necessary, add 1 drop of normal butyl alcohol to break the bubbles. This mixture should be flowed over an accurately leveled 8 x 10 in. glass and, when the gelatin sets, it may be dried in an upright position.

Directions are given for applying the gelatin to glass, preparing the filters and variations in use of the filters.—*T. M. McMillan*.

### MICROTECHNIC IN GENERAL

**COBE, HERBERT M., and SCHOENFLE, A.** A practical and inexpensive method for fixation of paraffin sections to slides. *Amer. J. Clin. Path., Tech. Sect.*, 10, 31. 1946.

The solution recommended to replace the commonly used egg albumin mixture is made by dissolving 200 mg. of sodium or potassium dichromate in 1 liter of distilled water, adding 200 mg. of sheet or granular gelatin, boiling in daylight for 5 min., cooling and filtering. This solution keeps indefinitely and can be refiltered if it becomes cloudy. It is used as follows: float paraffin sections in the above solution (maintained 5 degrees below the melting point of the paraffin in a white enamel pan with a sheet of black paper on the bottom to detect wrinkles or air bubbles in the section) to flatten them out; bring a clean slide from beneath the solution to the section; adjust in position on the slide, and dry in an incubator or paraffin oven. Frozen sections are floated in this same solution unheated and placed on a slide with a teasing needle. They are then carefully dried over a Bunsen burner avoiding overheating.—*L. Farber*.

**LACOSTE, A., and DE LACHAUD, R.** Préparation et utilisation d'un nouveau milieu de montage d'observation et de conservation des coupes histologiques avec ou sans lamelles. *Bull. d'Histol. Appl.*, 20, 159-60. 1943.

Due to the difficulty of obtaining the usual mounting media in France, the authors were forced to develop a method for making a medium by dissolving resin in xylene or toluene. Their procedure is as follows: Place 120 g. of the most transparent resin in a muslin bag and dissolve this in 100 ml. of toluene. After solution, remove the bag which contains the large impurities and allow the remainder to stand for 24 hr. By decanting, a clear light yellow liquid is obtained which dries rapidly without cracking and does not alter the color of the dyes. The refractive index is 1.519 at 22° C., and the medium may be used with or without a cover glass.—*Jean E. Conn*.

SCHUSTER, MARY C., and FULLAM, E. F. Preparation of powdered materials for electron microscopy. *Ind. Eng. Chem., Anal. Ed.*, 18, 653-7. 1946.

A discussion is given of the basic problems involved in the preparation of powdered materials for electron microscopy. A number of methods are described for dispersing and mounting various types of materials.—*R. T. Whittenberger.*

### ANIMAL MICROTECHNIC

ALMQUIST, JOHN O. The effect of certain coal-tar dyes used for semen identification on the livability and fertility of bull spermatozoa. *J. Dairy Science*, 29, 815-20. 1946.

Certified coal-tar dye solutions were found safe to use as a practical means of coloring diluted bull semen to identify different breeds.

The following certified foods, drugs, and cosmetics liquid colors were found to give adequate color differentiation when added to diluted semen: (1) Strawberry Shade Red Color (pure dye content not less than 5.2%), (2) Emerald Shade Green Color (pure dye content not less than 2.7%), (3) Purple Shade Grape Color (pure dye content not less than 4.1%), and (4) Brown Color (pure dye content not less than 3.7%). These liquid colors contain two or more of the certified dyes commonly known as amaranth, ponceau SX, ponceau 3R, tartrazine, fast green FCF and brilliant blue FCF. The brown color also contains a substantial amount of caramel (burnt sugar color).

The liquid colors used in these studies were aqueous solutions of the various certified foods, drugs, and cosmetics dry colors and contain as a preservative against molds, 0.1% sodium benzoate and enough food grade phosphoric acid to lower the pH to approximately 3.7.

Spermatozoa in diluted semen, colored by adding one drop of the basic red, green, brown, and purple dye solutions to each 10 ml. of diluent, maintained their motility as well as did those in the uncolored controls.

In a field study, when the same dyes were used at a lower concentration, no significant differences in fertility were found between the colored and uncolored semen samples. This investigation involved 2,995 inseminations and an average of 59.9 cows were inseminated per semen sample.—*William G. Walter.*

FEENEY, JAMES F., JR., AND WATTERSON, RAY L. The development of the vascular patterns within the walls of the central nervous system of the chick embryo. *J. Morph.*, 78, 231-304. 1946.

After injection of undiluted India ink into selected veins, chick embryos were fixed from one day to one week in Bouin's fluid, washed in several changes of 70% alcohol (presumably ethyl alcohol) to remove picric acid, transferred to 85% alcohol, and then into Mayer's alcoholic HCl carmine stain, diluted one-half. After a bath in this stain, ranging from 2 days to a week, the embryos were washed in several changes of 95% alcohol to remove excess stain. They were then placed in 100% alcohol for at least two days, into equal parts of 100% ethyl alcohol and anhydrous ether for one week. They were successively infiltrated for one week in each of 10%, 25%, and 50% nitrocellulose, and finally embedded in nitrocellulose.—*Elbert C. Cole.*

GLUCKMAN, F. Sur une méthode de détection rapide, à chaude, des cellules argentaffines (adrénalinipares) de l'intestin. *Bull. d'Histol. Appl.*, 20, 63-4. 1943.

Masson's silver impregnation method for intestinal tissue has several drawbacks due to the slowness of the procedure. The author proposes a method which increases the speed of the reaction by raising the temperature. His technic is as follows: To 15 g. AgNO<sub>3</sub> in 100 ml. distilled water, add NH<sub>4</sub>OH, 50 ml., until the precipitate has completely dissolved. Remove the paraffin from thin sections of Bouin-fixed material and immerse in distilled water. After several minutes, transfer the slides to the silver solution which is held at a temperature of approximately 60° C. on a hot plate. After 5 or 10 min., remove the sections and place in sodium hyposulfite for 20 or 30 sec. Rinse in water for 2 or 3 min. Impregnation may be discontinued after 5 min., but the results are clearer if it is continued for 10 min.—*Jean E. Conn.*

LOPEZ, J. BARBEITO. A new staining method for histologic sections. Preliminary report. *Amer. J. Clin. Path., Tech. Sec.*, 10, 53. 1946.

The author proposes a technic which calls for the following solutions: (1) Fixative: 10% aqueous formalin solution. (2) Acetic fuchsin solution: to 100 ml. distilled H<sub>2</sub>O add 10 ml. of Ziehl's fuchsin solution and 0.2 ml. glacial acetic acid. (3) Acetic formal solution: to 100 ml. distilled H<sub>2</sub>O add 4 ml. of formalin and 0.2 ml. glacial acetic acid. (4) Aniline-blue-methyl-orange solution: to 100 ml. distilled H<sub>2</sub>O in a 250 ml. Erlenmeyer flask add 1 g. of molybdic acid; heat gently 5 min.; add 0.5 g. aniline blue W.S. and shake to dissolve; add excess methyl orange so that some remains undissolved, and filter.

The following staining procedure is recommended: fix 24 hr. or more; embed in paraffin and section; deparaffin sections; stain 30 sec. to 1 min. in solution 2; wash vigorously in tap water to remove excess solution 2; fix color by immersion in a Coplin jar containing solution 3 until color changes to a pale violet, usually requiring 3 min.; wash in tap water; stain 30 sec. to 1 min. with solution 4; wash rapidly in tap water; dehydrate with 95% and absolute alcohol; clear with xylene and mount in Canada balsam.

Two tissues have so far been used and their staining reactions are as follows:

*Kidney:* nuclei, violet red; cytoplasm of convoluted tubules, emerald green; cytoplasm of excretory tubes and papillary ducts, pale blue; reticulum fibers, deep blue; collagen, brilliant green; cytoplasm of capsular elements, deep violet-blue; epithelial lining of renal pelvis, pale violet-blue with fine granular cytoplasm; red blood cells, brilliant orange.

*Liver:* nuclei, violet-red; capsule and epithelium of bile ducts similar to cells lining renal pelvis; cytoplasm of glandular elements, greenish blue with purple-red pigment granules; reticulum, deep blue; fibrin, greenish blue; fibroblasts, green; Kuper cells, bluish color.—*L. Farber.*

THOMAS, J. A. L'hématoxyline phosphomolybdique au dioxane, colorant électif rapide des structures collagènes et réticulaires. *Bull. d'Histol. Appl.*, 20, 49-58. 1943.

The authors have found that a hematoxylin lake can be prepared which is stable and ready for use and will stain collagenous and reticular structures an intense violet in several seconds. The technic is simple and gives clear and selective results. The lake is obtained by the reaction of aqueous phosphomolybdic acid with hematoxylin dissolved in dioxane. It is prepared as follows: Mix, in equal parts, Solution 1 (49 ml. dioxane, 1 ml. aerated water, 2.5 g. crystalline hematoxylin) and filtered Solution 2 (44 ml. distilled water, 11 ml. diethylene glycol, 16.5 g. phosphomolybdic acid). The lake forms rapidly but it is better to wait until the next day before using it. If the diethylene glycol is omitted, the mixture is ready for use after an hour.

The authors recommend two staining procedures. The first is a simple stain: Treat the deparaffined section with the above lake for 1 or 2 min. Wash in distilled water. Differentiate several seconds in picro-acetic alcohol (0.5 g. picric acid, 0.5 ml. acetic acid, 100 ml. 70% alcohol). Dehydrate as usual and mount in cedar oil or balsam. The second is a trichromatic procedure: To stain the nuclei, treat with ferric hematoxylin in dioxane for 30 sec. or hemalum I for several minutes. Wash in water and differentiate in 70% alcohol containing 0.5% HCl. Stain the collagenous and reticular structures with phosphomolybdic hematoxylin in dioxane for 30 sec. or 1-2 min. Wash in distilled water and differentiate in picro-acetic alcohol. Stain the cytoplasm with acridine yellow dissolved 1:5000 in distilled water containing 1% acetic acid. Rinse in distilled water and dehydrate with alcohols, not dioxane. Mount in cedar oil. The chromatin is black or bluish-black; collagen and reticulin, deep violet; cytoplasm, green, yellow green or yellow brown.—*Jean E. Conn.*

#### PLANT MICROTECHNIC

SHAPIRO, SEYMOUR. Warm safranin for plant tissues. *Science*, 105, 50. 1947.

A method for staining plant tissues with safranin solution at 53° C. is described with results equal to those obtained by the much longer standard method. The

procedure before and after using the warm safranin is as usual. Satisfactory staining may be obtained in 15 min., and may be used up to  $3\frac{1}{2}$  hr. The heat induces a thorough penetration of the dye and excess stain is not as readily removed as under room temperature.—*T. M. McMillion.*

### MICROÖRGANISMS

**QUANDT, JOCHEN.** Erfahrungen mit der karbolnachtsblaufärbung der Tuberkelbazillen im sputum und gewebe. *Zentbl. Bakt.*, I Orig., 151, 348–50. 1944.

A method for staining tubercle bacilli in sputum and tissue slices is described. The staining solution is prepared by adding immediately before use 10 ml. of 5% night blue in 95% alcohol to 90 ml. of the following solution: 2.5 ml. melted phenol; 0.2 ml. 10% KOH; 100 ml. distilled water. The destaining solution consists of 3 ml. 25% HCl in 100 ml. 70% alcohol. Chrysoidin, 2 g. in 300 ml. distilled water, is used as the counterstain. The technic is that of the Ziehl-Neelsen procedure.

When this method is used, the bacilli can be easily distinguished from the stroma and tissue background.—*C. Randall.*

### HISTOCHEMISTRY

**BAUER, KARL, FRIEDR.** Beitrag zur Frage der "Silbergranula" im Cytoplasma. *Zts. Wis. Mikr.*, 59, 142–52. 1943.

According to Giroud and Leblond the presence of vitamin C may be demonstrated by injecting intravenously a silver nitrate solution, acidulated with acetic acid, and letting it act for a short time. The dark, intracellular granules produced represent the silver salt reduced by ascorbic acid. The author is in agreement with these findings; he calls attention, however, to the appearance of intracellular silver granules by various other technics. These granules are said to be morphologically identical with the vitamin C granules. He concludes that either vitamin C granules can be demonstrated by various methods or else silver granules can be produced in the cytoplasm which bear no relationship to vitamin C.—*J. M. Thuringer.*

**TUBA, JULES, HUNTER, GEORGE, and OSBORNE, JOHN A.** On staining for Vitamin C in tissues. *Canad. J. Res., Sec. C*, 24, 182–7. 1946.

To determine the histological distribution of vitamin C in rose hips, the staining solution of Smyth, Bingley, and Hill was used, consisting of 5 ml. 5% acetic acid and 100 ml. 5%  $\text{AgNO}_3$ . In a normal guinea pig with a high content of ascorbic acid, numerous fine black granules appeared in the adrenals when stained but in scorbutic animals few such granules were present. Numerous granules were found in leaves of the moss species *Mnium cuspidatum* and *Mnium affine* but they were not associated with the chloroplasts. Although guinea pig adrenals and moss leaves can be stained readily because the stain penetrates animal tissue readily and moss leaves are very thin, the following method was necessary for rose hips: Embed fresh rose hip in paraffin and cut away top third on a sliding microtome; immerse block in staining solution; leave in the dark at room temperature for 30 min.; wash block with sodium hyposulphite solution; remove excess moisture; section; mount sections in Farrant's solution. Young hips should be sectioned at  $15\ \mu$  and old ones at  $45\ \mu$ . In young hips of *Rosa acicularis* the ascorbic acid content was low and vitamin C was found to be definitely associated with the chloroplasts. As the hips mature, the vitamin C increases and accumulates in cells outside the chloroplasts. The density of staining roughly parallels the content of ascorbic acid.—*H. P. Riley.*

the best, such preparations could be used for teaching purposes. In research work where every animal counts, the failures might prove to be so numerous that it would seriously handicap the evaluation of results. This is not true of nerve trunks where stains are obtained with a considerable degree of regularity.

Three different lots of protargol were used (all Winthrop) and no differences noted in their behavior. This reagent is we believe the best in current use for silver-on-the-slide staining technics. The low percentage of successes when staining intestinal nerves need not influence its use for more suitable tissues.

## APPENDIX

The following procedures are suggested as those most likely to give stains of nerve fibers in the intestinal wall.

### FIXATION

Fix for 24-48 hours in either of the three fluids listed below, or preferably fix one specimen in each.

1. Formalin, 10 ml.; glacial acetic acid, 5 ml.; picric acid, saturated aqueous solution, 50 ml.; ethyl alcohol, 40 ml. (Bouin's fluid with alcohol)

2. Saturated aqueous picric acid, 50 ml.; formamide, pure (Eastman Kodak Co.), 10 ml.; alcohol, 40 ml.

3. Formamide, 10 ml.; paranitrophenol, 5 g.; pyridine (Merck's medicinal), 5 ml.; 40% alcohol to make 100 ml.

Dehydrate, embed in paraffin and cut and mount in the usual manner. Fixative No. 1 may be expected to give the best fixation but least vigorous staining. No. 3 has given us some of the best differentiated nerve fibers within the circular layer of muscle, but fixation is poor. No. 2 is intermediate.

### STAINING

Any of the published protargol technics for nerve fibers would seem to have about an equal chance for success. The only new variation incident to this research is the use of aniline blue or fast green FCF mixed with the protargol. Both of these dyes appear to effect some suppression of smooth muscle and connective tissue staining without interfering with the staining of nerve fibers.

### FAST-GREEN-PROTARGOL METHOD

1. Remove paraffin with 2 changes of xylene, then absolute alcohol, 95% alcohol to which 1% ammonia water has been added (this removes the picric acid when present and does no harm if it is not present), 95% alcohol, 70%, 50%, and 2 changes of distilled water.

2. Stain in the following mixture at 37-40°C. for 24-48 hours (the 48-hour period may be needed if the stain is too light after 24 hours): Protargol (Winthrop), 0.5 g.; fast green FCF (or aniline blue W. S.) CC, 0.1 g.; distilled water, 100 ml.

3. Rinse the slides 2 or 3 seconds in distilled water and reduce in:  $\text{Na}_2\text{SO}_3$ , 5 g.; Kodalk (Eastman Kodak Co.) or potassium metaborate, 0.5 g.; hydroquinone, 1 g.; distilled water, 100 ml. Reduction for 2 min. is usually sufficient, but longer treatment is not harmful.

4. Wash thoroughly in tap water, preferably running water, to remove all of the reducing solution and rinse once in distilled water.

5. Tone in aqueous gold chloride solution of 0.2 to 1.0% concentration (uncritical) until the sections are gray.

6. Rinse and place in 0.2 to 0.5% oxalic solution. The stain usually darkens progressively in this second reducing process and care should be exercised to stop the action of the oxalic acid by the next step (hypo) before the darkening of the background obscures the neutral elements.

7. Rinse and place immediately in acid alum hypo made according to the standard photographic formula. Hypo alone (10% aqueous  $\text{Na}_2\text{S}_2\text{O}_3$ ) can be used but may cause some loss of the dye component of the stain. Fix about 1 min. and wash thoroughly in water.

8. Dehydrate through grades of alcohol, each of which should be made slightly acidic by the addition of about 0.5% glacial acetic acid. The acidification assists in retaining the color.

9. Xylene, cover.

#### NOTES ON THE STAINING PROCEDURE

The protargol-fast-green stain gave good results on peripheral nerves after formamide-picric-acid fixation (No. 2) and formamide-paranitrophenol-pyridine (No. 3) or the latter with the pyridine omitted. It was less certain with us after the Bouin's type of fixation (No. 1), or other mixtures of formalin.

When aniline blue is used, its color fades out in the protargol if the pH is correct. This is an indicator effect and the color reappears in the gold chloride bath.

Either dye is usually somewhat degraded by the sulfite reducing solution. The fast green is less affected than aniline blue. Occasionally the tissue failed to retain the dye—reason unknown.

The dye used should be near neutrality since any excess of acidity or alkalinity may affect the stain. See articles by Holmes (1943) and Silver (1942) on the effect of pH.

#### ACKNOWLEDGMENTS

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## THE ACTION OF RIBONUCLEASE ON FIXED TISSUES<sup>1</sup>

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**ABSTRACT.**—To study the optimal conditions for histochemical use of ribonuclease on fixed tissues, the factors of (1) type of fixation, (2) temperature, pH, type of buffer and length of incubation, (3) concentration of enzyme, and (4) staining and dehydration of sections were observed on rabbit pancreas.

The fixing fluids studied were sublimate-alcohol, Bouin's, Zenker-acetic, Zenker-formol, Petrunkevich's cupric-paranitrophenol, 10% neutral formalin, SUSa, Carnoy, Bensley's chrom-sublimate, absolute ethyl alcohol and acetone. Formaldehyde was a satisfactory fixative, although others might be preferred for special purposes. Of the five buffers tested, McIlvaine's citric-acid-disodium-phosphate mixture was the most satisfactory, whereas veronal-acetate extracted considerable stainable cytoplasmic material. The optimum concentration of ribonuclease and length of incubation varied greatly after the 11 different types of fixation. For example, with ribonuclease buffered by McIlvaine's fluid, the intense cytoplasmic staining of formaldehyde-fixed tissues was removed by concentrations as low as 0.001 mg./ml., whereas, with sections fixed in Zenker's fluid some cytoplasmic staining persisted even after 3 hours in 0.2 mg./ml. Under the conditions employed the temperature and hydrogen-ion concentration during incubation were less important. Examples of non-specific action of ribonuclease were noted. Until the degree and optimum conditions of specific action have been more precisely established by further experiments, it is suggested that this histochemical reaction only be interpreted as a confirmatory test which is, under the best conditions, only relatively specific for ribonucleic acid and not highly quantitative.

### HISTORICAL REVIEW

The histochemical use of ribonuclease for determining the localization and relative amounts of ribonucleic acid is based upon the incu-

<sup>1</sup>Part of a paper presented by the senior author at the Symposium on the Biology and Biochemistry of Nucleic Acid, Cambridge, England, July 10, 1946.

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bation of tissue in solutions of a specific enzyme and the subsequent comparison of the staining of the incubated and control tissue with basic dyes. The accuracy of this histochemical test is dependent upon (1) the preservation without change of position or loss from the cell of the ribonucleic acid and related ribonucleotides during fixation and preparation of tissues for incubation, (2) the satisfactory specific action of the enzyme upon the substrate to destroy its stainability and the absence of such action at any time in strictly comparable control sections, (3) a permanent, precise dye which stains structures in intensity proportional to their content of nucleic acid, and (4) the satisfactory preservation of the stain during subsequent treatment. The second point mentioned presumes that the enzyme has free access to all of its potential substrate, and is not blocked by any non-permeable or inactive material or bound in some stable form to other substances, and furthermore that the diffusible reaction products are completely removed from the tissue.

The action of crystalline ribonuclease on fresh yeast nucleic acid has been described in detail by Kunitz (1940). Although ribonuclease, of varying degrees of purity, has been used by numerous workers on preserved tissues, only scant details of the specific conditions and mode of action have been given. Our own observations as well as personal communications from other investigators indicate that such technical details are of the greatest importance in obtaining even a relative specificity of enzymatic action on fixed tissues. The great diversity of the scant technical details which have been published, indicates that a more critical evaluation is desirable. Although our program of evaluating histochemical technics preparatory to their application in cancer research is of necessity temporarily interrupted, we feel that it is important to record briefly these observations on ribonuclease at this time.

The important role of nucleic acids and nucleoproteins in normal and pathologic tissues has been recently reviewed by Caspersson and Santesson (1942), Greenstein (1944), Davidson and Waymouth (1944d) and Stowell (1945). Numerous investigators have studied ribonucleic acid by the histochemical ribonuclease method. Brachet (1940a, b, c, d, 1945) employed a crude preparation of ribonuclease on tissues fixed in Helly's fluid. The fixed tissues were incubated for less than 1 hour at 65-70°C. and stained with methyl-green-pyronin. Brachet (1940d) recognized that the staining action of pyronin is not specific for pentose nucleic acids. Desclin (1940) applied a nuclease at 70°C. for 1 hour and stained pituitary tissue with methyl-green-pyronin stain. Painter and Taylor (1942, 1945) treated tissues for

several hours with crystalline ribonuclease and stained with Unna's methyl-green-pyronin mixture. Gersh and Bodian (1943) used a 1 mg./ml. concentration of crystalline ribonuclease in an acetate-veronal buffer of pH 6.75, incubated sections at 40°C. for 5 hours and then stained with toluidine blue. Davidson and Waymouth (1944a, b, c) employed fixation in formol-saline and essentially the same conditions for incubation and staining as Gersh and Bodian used. After incubating tissues for 2 hours at  $50 \pm 3^\circ\text{C}$ . in 0.2 mg. of crystalline ribonuclease per milliliter of McIlvaine's citric-acid-disodium-phosphate buffer at pH 7.0, Bieseke (1944) stained the tissues with Unna's carbol pyronin-methyl-green at 40°C. for 10 minutes. In the investigations of Roskin and Ginsburg (1944a, b), protozoa, which had been fixed in alcohol and treated with a ribonuclease extract for 90 minutes at 58–65°C., were stained with Unna's methyl-green-pyronin, Dominici or Giemsa stains. No technical details were given in later work with ribonuclease on normal and neoplastic tissue (Roskin and Kharlova, 1944c). Bartholomew and Umbreit (1944) used crystalline ribonuclease to convert Gram-positive organisms to Gram-negative ones, and subsequently Henry, Stacey and Teece (1945) found a ribonucleoprotein containing magnesium to which Gram-positive properties were attributed. In discussing this action of heat stable enzymes upon bacteria, Dubos (1945) points out that enzymes, such as lysozyme, with physicochemical properties similar to ribonuclease, may contaminate even crystalline preparations. Dempsey and Wislocki (1945) incubated tissues fixed in Zenker-formol, Zenker-acetic or Dawson's fluid for one-half to 3 hours at 60°C. and then stained them with eosin-methylene-blue. Control sections were incubated in distilled water for the same length of time, but the concentration of crystalline enzyme is not stated. Mottram and Selbie (1945), using plant and animal tissues, fixed in SUSa or Carnoy's fluid, stained them with Unna's stain or iodine gentian violet after ribonuclease treatment. Thyroid tissue, after fixation in Zenker fluid, was incubated in crystalline ribonuclease and then stained with eosin-methylene-blue or triacid "Azan" and Mallory stains by Dempsey and Singer (1946). Human, rat and mouse livers fixed in Zenker-formol were treated by Deane (1946) for 3 hours at 60°C. in a 0.1% ribonuclease solution buffered with sodium barbital to pH 6.75. The slides were stained with eosin-methylene-blue. Stowell (1946) used 0.1 mg. of crystalline ribonuclease per milliliter of McIlvaine's buffer at pH 7 in attempting to estimate ribonucleic acid content in tumors fixed in formaldehyde and sublimate alcohol, but the results were only roughly quantitative. This

brief resume shows that the technical details of the histochemical application of ribonuclease have varied greatly.

Following the discovery of the enzyme by Jones in 1920, there has been incomplete agreement regarding the exact action of ribonuclease even on fresh substrates. Kunitz (1940) regards his crystalline enzyme as capable of digesting and splitting yeast nucleic acid into fragments small enough to diffuse readily through colloidin or cellophane membranes. Eiler and Schmidt (1941) believe ribonuclease to be a specific phosphatase, but are unable to determine whether it hydrolyzes the linkage between nucleic acid and protein or one of the components of nucleic acid while the latter remains combined as nucleoprotein. Greenstein (1944) believes that most nucleases act by depolymerization. In reviewing the evidence on this controversy, Loring (1944) suggests that it is a phosphatase capable of breaking only some types of yeast nucleic acid linkages with the formation of mononucleotides. Therefore Loring (1944) and Zittle (1945) prefer the name ribonucleinase. The further elucidation of the properties of this enzyme will be aided by a better understanding of the structure of ribonucleic acid and nucleoproteins. Some of the differences in results may be explained by different preparations of ribonuclease<sup>4</sup> and by the presence of traces of impurities even in crystalline preparations. The basis for the specificity of nuclease action on fixed tissue, which certainly may differ from that of fresh tissue, has not been satisfactorily presented in the literature.

#### EXPERIMENTAL OBSERVATIONS

Using a variety of normal and pathologic mammalian tissues, such factors as (1) type of fixation; (2) temperature, hydrogen ion concentration, type of buffer and length of incubation; (3) concentration of enzyme; and (4) technic of staining and dehydration were studied in preliminary experiments to give the basis for the following experiments. Small pieces of adjacent rabbit pancreas, which contains large amounts of cytoplasmic ribonucleic acids, were fixed in 11 different preservatives, dehydrated in ethyl alcohol, cleared in chloroform (1 hour), infiltrated (2 hours) and embedded in paraffin, then cut in serial sections of  $6\mu$  thickness under comparable conditions. Tissue was fixed in (1) equal parts of 95% ethyl alcohol and saturated aqueous corrosive sublimate or (2) Bouin's fluid for 24 hours and rinsed and stored in 70% alcohol for 4 days before under-

<sup>4</sup>Most American workers, however, have obtained their crystalline ribonuclease from Dr. M. Kunitz of the Rockefeller Institute, Princeton, New Jersey, to whom appreciation is expressed for the ribonuclease used in these experiments.

going the standard dehydration and embedding. Blocks were preserved in (3) Zenker-acetic, (4) Zenker-formol, or (5) Petrunkevich's No. 2 cupric-paranitrophenol for 24 hours, washed in running water overnight, dehydrated and stored in 70% alcohol for 4 days. Pancreas was fixed in (6) 10% neutral formalin (4% formaldehyde) for 24 hours, rinsed in water and dehydrated in 70% alcohol. After 18 hours in (7) SUSa or 3½ hours in (8) Carnoy's fluid, tissue was washed in 95% alcohol overnight and stored in 70% alcohol 4 days. Tissue was fixed 18 hours in (9) Bensley's chrom-sublimate, washed quickly in water and dehydrated to 70% alcohol. After fixation in (10) absolute ethyl alcohol or (11) acetone for 24 hours, tissue was cleared in chloroform 1½ hours and embedded in paraffin. One section of each of the 11 blocks was mounted on each of 59 slides. Under comparable conditions, the tissues were treated with xylene, hydrated, treated with enzyme or control buffer solution or distilled water and then rinsed in distilled water. Some slides were stained for 20 minutes at room temperature with pyronin (Unna-Pappenheim solution<sup>5</sup> from which the methyl green had been deleted), rinsed rapidly in distilled water, dehydrated in tertiary butyl alcohol, cleared in xylene and mounted in damar. By deleting methyl green from the staining mixture, the uncomplicated action of pyronin was more readily studied. Staining at room temperature was found to be just as satisfactory as at elevated temperatures. Other sections were stained in a 1% filtered solution of toluidine blue for 18½ hours, dehydrated and differentiated in tertiary butyl alcohol, cleared in xylene and mounted in damar. The use of tertiary butyl alcohol permitted a more gradual dehydration and less rapid extraction of the dye.

The variables of temperature, length of incubation and concentration of ribonuclease were tested at 40°, 50°, and 60°C.; 3 hours, 1 hour and 30 minutes; and 0.001, 0.01, 0.1, and 0.2 mg./ml. respectively. Sorensen's borate-hydrochloric-acid buffer (Clark, 1928, p. 209), Sorensen's phosphate mixture (Clark, 1928, p. 210), veronal-acetate buffer (Michaelis, 1931), Clark and Lubs' potassium-acid-phosphate-sodium-hydroxide mixture (Clark, 1928, p. 200) and McIlvaine's standard mixture (Clark, 1928, p. 214) of buffers were compared at pH 7.95, 6.95, 7.05, 7.00 and 7.15 respectively. McIlvaine's buffers of pH 5.0, 6.0, 7.1 and 8.0 were likewise compared. In as far as possible conditions were adjusted to test only one variable at a time and slides receiving the same treatment were kept in the same or adjacent jars. Except when testing differences in buffers or

<sup>5</sup>Mallory (1938) p. 176.

hydrogen ion concentration, slides were incubated in McIlvaine's buffer at pH 7.0.

Great differences were noted in the staining of sections preserved in different fixatives. Tissue fixed in formaldehyde stained slightly heavier after treatment with McIlvaine's buffer than with distilled water. The intense cytoplasmic stain was completely prevented by ribonuclease even in concentrations of 0.001 mg./ml. The cytoplasmic preservation of formaldehyde-fixed tissue was, of course, only fair. In pancreas fixed in sublimate alcohol, the cytoplasm stained slightly less intensely and the nuclei more intensely after treatment in buffer control solutions than in distilled water. The ribonuclease, even in concentrations as low as 0.001 mg./ml. for 1 hour, inhibited cytoplasmic staining which was intense in control sections. Control sections of tissue preserved in Petrunkevitch's fluid stained slightly less heavily in buffer than in distilled water. The granular cytoplasm stained faintly with dye even after 3 hours in 0.1 or 0.2 mg. of ribonuclease per milliliter of buffer, whereas the nuclei stained more intensely. The nuclei of pancreas fixed in Bouin's stained negligibly with either pyronin or toluidine blue. The cytoplasmic staining, which was similar after control treatment with buffer or distilled water, was readily prevented by treatment with 0.001 mg. ribonuclease per milliliter for 3 hours and was only faint after 1 hour. Fixation of tissue in Bensley's chrom-sublimate gave sections in which nuclear and cytoplasmic staining was greater after control treatment with buffer than after distilled water. However, only moderate decrease in cytoplasmic staining was obtained after 3 hours in 0.1 mg. of enzyme per milliliter of buffer. The results on tissue fixed in Zenker-acetic or in Zenker-formol were quite similar and resembled those with Bensley's chrom-sublimate. Staining was slightly greater in buffer than water control sections and was only slightly reduced by treatment with strong enzyme solutions for 3 hours. The pancreas fixed in Carnoy, acetone or absolute alcohol was unsatisfactory since the faint to moderate cytoplasmic stain in distilled water control sections was absent in buffer controls. Nuclear staining in the buffer control sections was increased. The cells of *SUSA*-fixed tissue were not appreciably stained with pyronin or toluidine blue in any slides. Nuclear staining of tissue fixed in absolute alcohol or acetone was decreased appreciably by prolonged treatment in the higher concentrations of ribonuclease, indicating some non-specific action of the enzyme under the conditions employed. Decreased cytoplasmic staining was accompanied by increased nuclear staining in pancreas fixed in Carnoy, acetone, absolute alcohol or

sublimate alcohol. These observations were made upon the cells of the islets of Langerhans in which the cytoplasm stained negligibly or slightly in control sections, hence changes in nuclear staining were more noticeable than in the intensely stained acinic cells.

As expected from the above observations with different fixatives, the results with the other variable factors depend upon the fixation of the tissue. In comparing sections treated in different buffers with control sections in distilled water, it was evident that acetate-veronal buffer eliminated considerable stainable material in the absence of the enzyme. This action was more pronounced in those tissues in which cytoplasmic staining was easily prevented by low concentrations of ribonuclease. Clark and Lubs' potassium-acid-phosphate-sodium-hydroxide mixture and to a lesser extent Sorensen's phosphate buffer also had some tendency to decrease cytoplasmic staining in buffer control sections. McIlvaine's buffer permitted the best cytoplasmic staining and Sorensen's borate buffer the next best.

With ribonuclease in concentrations of 0.1 mg. per milliliter of buffer, acting upon sections for 1 hour, no significant differences in enzymatic activity were noted in McIlvaine's buffer ranging from pH 5 to 8. It is possible that slight differences might have been detected in weaker concentrations, since the hydrogen ion concentration is known to effect the enzymatic action on fresh yeast solutions (Kunitz, 1940).

Ribonuclease concentrations of 0.2 and 0.1 mg. per milliliter were equally effective at 3 hours, but not at 30 minutes. Solutions of 0.01 mg./ml. showed less effect upon cytoplasmic staining, but even 0.001 mg./ml. reduced staining considerably in some tissues, especially with prolonged incubation. With the higher concentrations of enzyme the nuclei of cells preserved in poor protein precipitants such as acetone tended to become distorted and in some instances were partially disintegrated. Sections fixed to glass slides with Mayer's albumin tended to be removed by treatment in solutions with concentrations of 0.2 mgm./ml. In preliminary experiments this loss of sections was prevented by coating the slides with dilute celloidin. These findings suggest a non-specific proteolytic action of the higher concentrations of the ribonuclease.

The length of incubation seemed to bear a direct relationship to the enzymatic action upon the cells. With concentrations of 0.001 and 0.01 mg./ml. it was evident that more stainable cytoplasmic material is extracted with longer incubation periods, but with concen-

trations of 0.1 mg./ml. or greater, no increased effect was noted with prolonged incubation.

Although the enzyme is less active at room temperature, incubation at 60°C. was not appreciably more effective than 50° or 40° in destroying cytoplasmic staining.

The observation of increased nuclear staining associated with decreased cytoplasmic staining suggested that some split cytoplasmic constituents might be adsorbed upon the nuclei to give an increased staining. However, it was not possible to increase nuclear staining of cells by treating them with solutions containing split products of 1% yeast nucleic acid in 0.1 mg. of ribonuclease per milliliter for 1 hour.

Since magnesium activates desoxyribonuclease (McCarty, 1946) an attempt was made to enhance the action of ribonuclease by adding .003 molar magnesium sulfate to the solution. No changes were noted after incubation in 0.1 mg. ribonuclease per milliliter for 1 hour.

Toluidine blue was as satisfactory, if not more so, than pyronin as a stain. Under the conditions employed the intensity of stain in the cytoplasm was slightly less and in the nuclei slightly greater than with the pyronin. Perhaps because of the initially intense nuclear stain, increased nuclear staining associated with decreased cytoplasmic staining in some tissues was slightly less evident with toluidine blue.

#### DISCUSSION

It is hoped that the results of these experiments will provoke more critical study of the action of ribonuclease upon tissues. Although the reported results were confirmed in several experiments, it has not been possible to test all of the variables. The results with Zenker-fixed tissue were not outstanding, but the concentrations of ribonuclease were somewhat lower than employed by some authors. The activation of enzymes of the pancreatic tissue was not an important factor in the observations because comparisons were made with control material in both buffer and distilled water. Zittle (1945) has shown that the ribonuclease activity can be inhibited by mononucleotides. It would seem advisable for each investigator to determine carefully the optimum conditions for his own experiments and to use as pure enzyme as possible in the minimal concentration necessary to give the optimal effect. It is possible that ribonucleases from different sources might even vary in their action, since there is evidence that such is the case for desoxyribonucleases (Catchside and Holmes, 1946).

Four basic requirements for the specific histochemical use of ribonuclease were outlined in the introductory paragraph of this paper. Let us evaluate the evidence on these points. The preservation without change or loss of ribonucleic acid from the cell during the technical procedures preparatory to incubation is essential. However, it is recognized that soluble nucleotides in the cytoplasm of cells, which usually comprise a relatively small proportion of the total nucleotide content, are ordinarily removed from the cells by histologic procedures. Since the ribopolynucleotides may be liberated from cytoplasmic particles such as mitochondria or even completely removed from the cell by certain fixing agents, drastic technical procedures should be avoided. There is inadequate evidence that nucleotides may be adsorbed onto other cellular structures, yet the possibility must be admitted. Although the major part of the ribonucleic acid of cells may be preserved satisfactorily within the cell during carefully controlled technical procedures, there is a possibility of some loss or shifting of position.

A second essential for this histochemical technic would be its specificity. Ribonuclease may have a specific action on fresh solutions of yeast nucleic acid. However, the reaction of enzymes on fixed cells is much more complicated than in simple homogeneous solutions. The evidence presented here of loss of nuclear as well as cytoplasmic staining after certain fixations, of disruption of nuclear membranes, and of removal of sections from slides suggests that there is only a relative specificity of enzymatic action on fixed tissues. The fact that control buffer solutions remove the stainable cytoplasmic material after fixation in Carnoy, acetone, and absolute alcohol shows how non-specific the removal of such material can be and emphasizes the importance of proper controls. Impurities present even in crystalline preparations may alter the action of ribonuclease and the use of crude extracts should be avoided. As Danielli (1946) has suggested, it is important to know that an enzyme is able to react with all the available substrate and that the diffusible reaction products are satisfactorily removed. The evidence does not prove that ribonuclease has a highly specific action upon tissues.

For a satisfactory histochemical technic, it was further suggested as desirable that the intensity of dye be proportional to the content of nucleic acid in the stained structures and that the quantity of stain should not be affected by subsequent technical procedures. A variety of stains has been used in conjunction with ribonuclease to detect nucleic acids in cells, but none is specific for ribonucleic

acid and the amount of stain present can be considered only very roughly proportional to the nucleic acid content. Some of the stains which are employed are readily removed by the dehydrating agents usually employed or, as in the case of pyronin, fade upon prolonged exposure to light. These factors introduce additional sources of error in quantitative estimations.

When comparative measurements are made upon the same section of tissue some of the errors may be reduced, but with our present limited knowledge and technics it would seem advisable to use the ribonuclease technic only crudely to confirm observations made by other methods. The results of these experiments do not prove that ribonuclease could not act specifically upon tissues under certain carefully controlled conditions. They do definitely indicate that under certain circumstances the enzyme is not specific and that additional work is needed to establish the degree of specificity and optimum conditions of action on tissues.

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## A CONFIRMATION OF RAFALKO'S FEULGEN METHOD

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**ABSTRACT.**—A method nearly identical with that used by Rafalko on small amoebae, oocyte prophases of *Habrobracon* and several yeasts, has been found confirmatory to his results when applied to mammalian testicular tissue. The method is described, additional preparational notes are given, and several questions raised on possible improvement of the technic.

Several years ago the writer used a Feulgen method very similar to that recently described by Rafalko (1946), the results of which can now be seen to be sufficiently confirmatory to those reported by him as to suggest that it may constitute a fundamental improvement in the technic, for reasons of both simplicity and efficiency. In both cases the method of preparing reagents is based on a procedure in common use in chemical laboratories (Shriner and Fuson, 1940), in which the production of sulfurous acid is separated from the production of sulfur dioxide.

In preparing reagents, methods which were used were essentially similar to Rafalko's. Fuchsin-sulfurous-acid (leucobasic fuchsin, bis-*n*-amino sulphonic acid, decolorized fuchsin, fuchsin aldehyde reagent, Schiff reagent, etc.) was prepared as follows: 0.1 g. basic fuchsin was dissolved in 100 ml. of water and filtered; 100 ml. of sulfurous acid was added, mixed, and allowed to stand for about 15 hours under refrigeration and in absence of light.

The sulfurous acid may be purchased (C. P. grade; concentration of SO<sub>2</sub> ranging from 6 to 8% by weight) or may be prepared by saturating distilled water with sulfur dioxide. Sulfur dioxide may be obtained as cylinder gas or may be produced through action of a dilute acid (sulfuric or hydrochloric) on a saturated aqueous solution of any sulfite, delivering the gas from the generating flask to the receiving flask (containing the water to be saturated) as it is formed. Saturation is usually performed at room temperature and carried to excess; if it is desired, the solubility of sulfur dioxide may be increased by chilling the sulfurous acid solution to 5 or 10°C. If it is considered necessary or advantageous to do so, the gas may be water-washed by sending it through one, or a series of several connected receiving flasks before using it in the final concentration.

Precise measurements of acid and sulfite are not required when

this method is used, and neither reagent need be of C. P. grade. As indicated, dilute solutions of either hydrochloric or sulfuric acid may be used, but nitric acid should be avoided unless used in very dilute solutions, perhaps not more than 2%; otherwise the sulfite may be oxidized to a sulfate without the liberation of sulfur dioxide. The source of sulfur dioxide may be a sulfite, a bisulfite, a metabisulfite (=pyrosulfite) or a hyposulfite (=hydrosulfite), although the most convenient compound is probably potassium metabisulfite ( $K_2S_2O_5$ ), as recommended by DeTomasi (1936). Various authors have referred to the bisulfite ( $-HSO_3$ ), the metabisulfite ( $-S_2O_5$ ), and sulfur dioxide ( $SO_2$ ) as being the reducing agent of basic fuchsin; the actual reducing agent of basic fuchsin, in preparing the Schiff or Faelgen reagents, is the water solution of sulfur dioxide, i.e. sulfurous acid ( $H_2SO_3$ ).

The staining schedule used was as follows:

- (1) Run down to water.
- (2) Distilled water at room temperature, 1 hr.
- (3) *N* HCl at room temperature, rinse.
- (4) *N* HCl at 60°C., 10 to 15 min.
- (5) *N* HCl at room temperature, rinse.
- (6) Distilled water at room temperature, rinse.
- (7) Fuchsin-sulfurous acid, 2 hr.
- (8) Sulfurous acid, 3 changes of 10 min. each.
- (9) Distilled water, 3 changes of 20 min. each.
- (10) Dehydrate through ethyl alcohol, clear through xylene, and mount.

I have as yet had no occasion to use the modified schedule described by Rafalko (1946). Tests on mammalian (*Peromyscus t. truei* and *P. n. nasutus*) testicular tissue, fixed with B-15 and dehydrated with dioxane, were confirmatory to Rafalko's results on small amoebae, oocyte prophases of *Habrobracon*, and several yeasts. Staining was more sensitive and precise than when reagents were prepared by the more customary methods. Interphase as well as divisional stages were stained in both mitotic and meiotic cells, and there were fewer general failures of staining. The color of the chromatinic elements was violet with a faint pinkish tinge.<sup>1</sup> As may be noted, no counterstain was used.

At least three points are still open to experiment with respect to further improvement of the technic:

<sup>1</sup>Dye lot: product of National Aniline and Chemical Co., Inc.; Stain Commission Certification No. NF 31; total dye content 89%.

# STAIN TECHNOLOGY

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## PROGRESS IN THE STANDARDIZATION OF STAINS

### A NEW LOCATION FOR THE CHEMICAL WORK

A year ago<sup>1</sup> announcement was made in these pages of the end of the coöperation with the U.S. Department of Agriculture, through whose agency the chemical and spectrophotometric work of the Stain Commission had been carried on in the past. Immediately after this relation came to an end, the chemical work was put temporarily in the hands of a firm of analytical chemists, while the spectrophotometric analyses were transferred to Geneva.

Gradually the work carried on at Geneva has been increased in scope until it has come to include not only the chemical assay of samples submitted but a broad field of chemical and spectrophotometric research. This work has been under the supervision of Dr. E. H. Stotz, who is the representative on the Commission of the American Chemical Society; and his interest in the field investigated has proved of much value to the Commission.

Now it is necessary to move the work again; but fortunately in so doing the assistance of Dr. Stotz will be retained. He has just left the New York State Agricultural Experiment Station to accept a position as head of the Department of Biochemistry at University of Rochester Medical School. He is taking with him the chemical and spectrophotometric work of the Commission, primarily as a research program to be undertaken in the Department of which he is to be in charge; but it will be possible for him to include with the research work, the routine assay of such samples as are submitted to the Commission for testing. This work is to be financed by means of a grant made by the Stain Commission to the University of Rochester.

It is felt that this will be a very desirable arrangement from the standpoint of the Commission. It was understood from the beginning that the location of the chemical work at Geneva was only temporary. Nevertheless, although this arrangement has continued less than two years, it has enabled us to get the chemical and biologi-

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<sup>1</sup>Conn, H. J. 1946. Twenty-five years of cooperation with the U. S. Department of Agriculture. *Stain Techn.*, 21, 81-6.

cal work knit together in a closer fashion than ever before, and there is no reason why the two lines of investigation cannot continue in the future in as close harmony as has been the case this last year.

As a matter of fact, some of the biological work will now be carried on at Rochester, also; for Miss Mary A. Darrow, who has been a valued employee of the Commission for some time, is also moving to Rochester and will continue her work for us there. This latter change might cause some inconvenience but for the fact that Rochester and Geneva are so close together that the work will remain in almost as close touch with the Commission headquarters at the latter place as it has been in the past.

Under this new arrangement, it is planned to carry on at Rochester a considerable program of research, largely concerning reasons for variations in the chemistry and optical properties of various samples of any dye, and the relation of such variations to their staining properties. Coöperation of other departments of the Medical School at Rochester has been promised; and it is hoped that the research can, therefore, cover a broader field than would be possible if it were continued at Geneva.

#### GRANTS FOR RESEARCH WORK

Elsewhere in these pages last year (p. 42, April, 1947) a brief note was made of a grant to Northwestern University Medical School for investigations in neurological microtechnic under the supervision of Dr. H. A. Davenport. This is proving a prolific bit of research; two papers have already been published, one in the April number of this journal, the other in the current issue.

The papers already published have dealt with the stainability of nerve fibers by protargol and by methylene blue, respectively; both of these fields of investigation are being continued. In addition to this program, Dr. Davenport reports his intention of investigating the Feulgen reaction as a possible block staining method for general histological purposes. The grant made for this work in 1946 has been renewed this year, thus extending the program into 1948.

Another such grant, in a different field, is now under consideration.  
—H. J. CONN.

## DIFFERENTIAL CYTOPHYSIOLOGICAL DIAGNOSIS OF CANCEROUS AND NORMAL TISSUES

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**ABSTRACT.**—A method is offered for the differential diagnosis of cancer cells. It depends on the use of methylene blue decolorized with sodium thiosulfate (denoted here HLM, i.e. "hyposulfite methylene blue"); this is prepared by dissolving 800 mg. sodium thiosulfate in 10 ml. of 0.1% aqueous methylene blue and adding 3-5 drops of dilute (1:3) HCl. Frozen sections are treated with this reagent for 2-3 minutes, rinsed with a large amount of distilled water, then stained 2-3 minutes with 0.05% aqueous acid fuchsin. Staining should be performed in a darkened room. If all due precautions are observed, normal tissue appears blue, malignant tissue red.

According to Unna (1916), the nuclei of most diverse normal tissues are stained intensely blue with the leucobase of methylene blue. By means of this leucobase (Rongalitweiss) a peculiar phenomenon was found, viz. in normal cells the cytoplasm stains light blue and the nuclei dark blue, while in the cells of malignant tissues, the cytoplasm either remains colorless or assumes a light bluish tint, and the nucleus as a rule remains colorless (Roskin, 1931, 1932, 1937; Roskin and Solowjewa, 1935). This phenomenon was confirmed by Waterman (1934) and Voinov (1940). It seemed the more interesting as the positive reaction with Rongalitweiss depends on the presence, in the normal cells, of an enzyme (an oxidoreductase). That is to say, we are dealing here not merely with microtechnical staining of the common type, but with a peculiar cytophysiological staining reaction of normal and malignant cells.

This conclusion is further supported by the fact that the staining reaction of normal cells with Rongalitweiss is prevented by heating to 80° C. for 30 minutes, as well as by alcohol, formol or treatment with KCN (*M*/1000) for 40 minutes or by a 30 minute exposure to ultraviolet. Of course, it is easy to show that none of these factors exert any apparent effect upon the common staining of the cells with methylene blue. Thus, a negative reaction of malignant cells to Rongalitweiss not only indicates its cytophysiological peculiarity but may also be used for differential diagnosis.

It is worth mentioning that the nuclei of many Protista, unlike those of the Metazoa, show a staining reaction similar to that of malignant cells. This fact may prove of interest for further study (Roskin and Semenov, 1933).

The application in this way of Unna's Rongalitweiss for cytodiagnostic purposes may, however, involve certain difficulties. A new

technic has therefore been developed in which the hyposulfite (i.e. sodium thiosulfate) leucobase of methylene blue (HLM) is used as the basic reagent. We believe that the new method secures rapid differential staining of normal and malignant cells and tissues. The HLM method may prove more than an aid in the routine histopathological practice, especially for diagnosis of malignant cells in exudates and punctates. Indeed, it may become a useful tool for studying the properties of cells in diverse forms of malignant and benign tumors, in precancerous states, etc. The value of this method of cytodiagnosis of malignant tumors has been checked on extensive material of the Moscow Oncological Institute, and Dr. Voinov (Thesis for a Doctor's degree, 1941, personal communication) has obtained very useful results in over 300 cases of diverse malignant and benign tumors.

#### PREPARATION OF THE HYPOSULFITE LEUCOBASE OF METHYLENE BLUE (HLM)

To prepare the leucobase of methylene blue, the following mixture is used: 800 mg. sodium thiosulfate, ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ) 10 ml. of 0.1% aqueous methylene blue and 3-5 drops of hydrochloric acid (sp. gr. 1.17) diluted 1:3 with distilled water. The staining results are best with 4 drops of HCl; with less acid the leucobase becomes unstable and is soon transformed into a light blue solution which is no longer good for staining. The methylene blue solution is prepared with redistilled water and is thoroughly filtered before use.

Sodium thiosulfate should be chemically pure (C. P. grade); the commercial "hypo" should be dissolved in distilled water, filtered and recrystallized, the dry crystals being kept in a tightly stoppered jar.

In preparing the leucobase the following directions should be strictly observed: 800 mg. sodium thiosulfate are put in a clean flask (this should be absolutely clean and dry) to which 10 ml. 0.1% aqueous methylene blue are added. As soon as the thiosulfate is completely dissolved (shake several times) 4 drops of diluted HCl are added to the flask. The solution is agitated while being carefully heated over a burner (20-30 seconds) until the appearance of a gray-milky sediment. The solution is then cooled (5-10 minutes) and thoroughly filtered through a double filter. The funnel, like the vessels generally, should be absolutely clean with no traces of methylene blue; otherwise the filtered leucobase would become blue. If the filtered leucobase assumes a blue tint, it should stand for a while in the dark. The methylene blue leucobase should be prepared not long before use but it can be kept in the dark for 10-12 hours. The properly prepared leucobase is colorless or slightly yellowish. Only such a leucobase is suitable for staining sections.

## PREPARATION OF SECTIONS

The sections are prepared by means of a freezing microtome from fresh non-fixed tissue fragments. The thickness of the sections is 10–15  $\mu$ . Pieces of normal or pathological tissues may be kept before use in a refrigerator ( $-2$  to  $+2$  C°.) in physiological saline for a period of 1–2 days.

The sections are transferred to physiological saline (in a Petri dish) and from there, by means of a pipette, to a slide (excess of physiological saline is sucked off with filter paper). They are then spread over the slide by means of needles and dried at room temperature. Beside the section of a tumor or other pathological tissue, a section of normal tissue is attached to the slide (e.g. of kidney or heart of a mouse) and serves as control of the staining effect. Such dried sections are ready for staining, either immediately or one or two days thereafter. The dried sections of malignant tumors preserve their typical staining capacity for two weeks provided they are kept in a dry and dark place.

## STAINING OF SECTIONS

The leucobase solution is pipetted onto a slide with sections attached. This leucobase should remain colorless throughout staining. If the leucobase solution becomes bluish, it should be changed. Staining should be continued until the section of the normal tissue stains a distinct blue while the section of the malignant tissue remains colorless—usually not more than 2–3 minutes. The leucobase is then poured off, the sections rinsed with a large amount of redistilled water after which the section is treated with 0.05% aqueous acid fuchsin (fuchsin S) for 2–3 minutes. Overstaining should be avoided. The acid fuchsin is then poured off and the sections well dried. The sections may be examined in glycerol or embedded in glycerol-gelatin. Permanent preparations may also be made by embedding the dried sections in pure paraffin oil and sealing the cover slip with a proper medium (asphalt varnish, etc.). The sections may also be embedded in Canada balsam but this should be neutral; otherwise the sections of cancerous tissues soon become blue.

Staining with the leucobase of methylene blue requires strict illumination conditions; the whole staining process should be carried out in a darkened room. Bright illumination may distort the staining effect as the leucobase solution is changed by light. Finally, we repeat: to avoid erroneous results of staining, the sections should be stained only with a colorless solution of the leucobase. With the proper observance of the above directions the difference between

malignant and normal tissue can also be observed macroscopically, viz. normal tissue appears blue and malignant tissue red.

The above HLM method is applicable not only to sections, but also to smears of various normal and pathological tissues.

#### • RESULTS OF STAINING

The nuclei of normal cells stain dark blue while the nuclear structures become quite distinct; the nuclei of cancerous cells either remain almost colorless or assume a diffuse hardly noticeable light blue or a slightly perceptible pale pink color. The nucleoli of malignant cells appear more intensely colored than the almost colorless nuclei although their blue color is far below that of normal nucleoli. The protoplasm of normal and malignant cells is stained distinctly red by fuchsin S. The conclusion is thus indicated that in contrast to the nuclei of normal cells those of malignant cells are unable to transform the colorless hyposulfite leucobase of methylene blue into the colored form.

A combination of this method with that of MacCarty (increased size of nucleoli of cancer cells) may presumably yield rapid and relatively reliable results. See MacCarty (1928, 1929), MacCarty *et al.* (1933, 1934).

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## SOME SYNTHETIC RESINS IN COMBINED FIXING, STAINING AND MOUNTING MEDIA

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**ABSTRACT.**—Many of the recently devised plasticizers and resins can be utilized to advantage in cytological technics. Some of them have solubilities which enable us to incorporate them in such fixing and staining solutions as aceto-carmin and propionic-carmin. They are non-volatile, do not alter the fixation images of the fluids with which they are mixed, and serve as mounting media as the volatile components evaporate. Thus it is possible to make a permanent slide in a single operation. These newer compounds are better adapted for this technic than are the natural balsams which have been used previously, as their greater tolerance for water provides a much greater margin of safety. Procedures are described for the utilization of (1) Rezyl 7020, a water-soluble resin (now, unfortunately, not available), which dries to form a water insoluble film, (2) Amberol 750 and (3) Bakelite BR-7160, two alcohol soluble resins, more miscible in solutions containing water than are the natural balsams. Formaldehyde can be included in the aceto-carmin and propionic-carmin fluids with the result that more nuclear detail is preserved. Lacto-gelatin has some valuable properties as a mounting medium and can be used when the specimen is stained with orcein. Carmin, which gives a permanent stain in Rezyl 7020, Amberol 750 and Bakelite BR-7160 fades in lacto-gelatin.

Several years ago (1937, 1940) the writer described a number of mixtures which could fix and stain chromosomes and also serve as mounting media when the volatile components evaporated. The object of devising such fluids was to make possible the transforming of smear preparations into permanent mounts with the least possible effort. The technic as described has had a limited success, but the margin of safety was small in every case, and the careful work which was often required to make a permanent slide in a single operation defeated the chief objective of the method.

The mounting media tested were of two types, water-soluble and fat-soluble. The former included dextrin, dextrose, gelatin, gum arabic, pectin, sorbitol and sucrose. The fat-soluble mounting media were Canada balsam, sandarac, and Venetian turpentine. The water-

soluble media, with the exception of gum arabic, could be incorporated in the usual aceto-carminic fixing fluids. The uses and limitations of these liquids have already been listed (Zirkle, 1937, 1940). It is sufficient to record here that it is difficult to add enough of the non-volatile component to an aceto-carminic fixative to serve as an adequate mounting medium without altering the fixation image and preventing the swelling of cells and chromosomes which is such a valuable aspect of acetic acid fixation. Several investigators (Sax, 1937, La Cour, 1941, 1947, Speese, 1945) have corrected this fault by fixing with aceto-carminic or aceto-orcin and using one or another of the mixtures simply as a mounting medium.

The method of dissolving Canada balsam, etc., in fixing and staining solutions which contain water has been described in detail by the writer (1940) and need not be repeated. The limiting factor here is the amount of balsam and water which these fluids can hold together in solution. As the mixtures must also give a satisfactory fixation image and dissolve enough of the dye to stain the chromatin, they have to perform a number of functions simultaneously. As every component must be in sufficient quantity to function adequately, yet not in a concentration which will interfere with the reactions of other reagents, there is very little leeway possible in the proportions of the several ingredients. Wilson (1945) has avoided the worst limitations of the method by fixing, staining and mounting in different steps and thus he has extended the utility of the procedure.

During the past two years many new plastics, plasticizers and synthetic resins have become available. It was thought that a number of these might have the solubilities which would allow them to be incorporated in fixing, staining and mounting media and that such mixtures could be used with a greater margin of safety than any which have previously been described. The idea was fully justified; in fact, nearly all the plasticizers tested could be added in sufficient quantity to aceto-carminic without altering the fixation image and thus durable liquid mounts could be made which would last, with normal precautions, for several weeks. Some of the plasticizers could be combined with gum kauri, a natural resin, and permanent mounts could be made requiring fewer precautions than are necessary when Canada balsam, sandarac or Venetian turpentine were employed. There is no need to record these procedures in detail, however, for several of the synthetic resins proved to be so satisfactory that there seems to be little need for the liquid mounts.

It should be emphasized that the primary object of a single fluid technic is that it be quick and easy and that it require no special

precautions. Of course the fixing, staining and the final mounts should be at least as satisfactory as those obtained by other methods, but the introduction of several steps into the process to make it fit difficult biological material sacrifices one of its chief advantages. Small changes in the composition of the fluid itself should be sufficient to extend its applicability to different types of cells.

The proportions of the several components in the following mixtures were derived empirically, the pollen mother cells of *Tradescantia* being used as test material. For other material it is possible that these proportions could be altered to advantage.

Only three of the synthetic resins which were investigated are described here. Each has its particular advantages and disadvantages. The introduction of orcein as a cytological stain by La Cour (1941) has made it possible to mount stained specimens which keep their color in a lactic-acid gelatin mixture in which carmine fades. As this medium has its own particular virtues, its description is included.

#### REZYL 7020<sup>1</sup>

Rezyl 7020 is a water-soluble alkyd resin which has the very special property of drying into a water-insoluble film. Its index of refraction is approximately 1.484 but may vary slightly from batch to batch. After completing the work with this product, however, it was discovered that Rezyl 7020 is no longer on the market. A letter from the American Cyanamid and Chemical Company states that its manufacture has been discontinued, at least temporarily, due to two factors: (1) one of the necessary ingredients could no longer be obtained and (2) no large scale demand for the products developed. There seems to be little prospect for its manufacture being resumed in the immediate future although it may become available later. The following abbreviated account of the Rezyl technic is printed with the hope that it may be useful either if Rezyl 7020 again becomes available or some other resin is found with like properties.

Rezyl 7020 is soluble and stable in mixtures of acetic acid and water, and can be added directly to aceto-carmin. Three parts of aceto-carmin to one of Rezyl makes a workable mixture but a sharper fixation image is secured by increasing slightly the amount of acid. As formaldehyde and acetic acid together preserve more than either reagent does when used alone, the incorporation of

<sup>1</sup>A product of the American Cyanamid and Chemical Corporation, 30 Rockefeller Plaza, New York 20, New York. The writer is indebted to Mr. J. M. Cochran of the Organic Chemicals Dept. for supplying this resin.

formaldehyde with acetic acid in the usual smearing technic results in the preservation of more nuclear detail. Mixtures of acetic acid and formalin (40% aqueous solution of formaldehyde) in various proportions will dissolve sufficient carmine so the fixing and staining may be accomplished simultaneously. The following mixture is recommended:

Rezyl 7020 .....	25 ml.
Glacial acetic acid.....	30 "
40% formaldehyde.....	70 "
Fe(NO <sub>3</sub> ) <sub>3</sub> ·9 H <sub>2</sub> O.....	0.3 g.
Carmine .....	To saturation

Specimens fixed in the above are firmer and more resistant to distortion than they are when formaldehyde is omitted. Of course the Rezyl need not be included and the fluid can then be used to advantage for temporary smears.

Orcein can be used in place of the carmine. Flaming improves the quality of the fixation and secures the characteristic swelling of the aceto-carmine method. Cells can be flattened by applying pressure to the cover glass but when this is done an extra drop or two of the fluid should be placed at the edges of the cover glass to replace the liquid lost before the preparation dries.

*Advantages:* The chief advantage of the Rezyl technic is its simplicity and the ease with which it can be incorporated into a familiar procedure. As moisture does not hurt the mount no precautions need be taken for drying even in the extremely humid air of seaside laboratories. Once the Rezyl has hardened the mount is almost as impervious to water as some of the fat-soluble media. The mounting medium itself may constitute as much as 25% of these mixtures, as compared with 5-16% of the other water-soluble media. This gives sufficient leeway for decreasing the Rezyl in fluids designed for very delicate material.

*Disadvantages:* The chief disadvantage in the use of Rezyl lies in the relative slowness with which it solidifies under a cover glass. In addition it is not very tacky and does not adhere to glass as strongly as balsam but if the smear is thin and enough of the medium has been used, no air need be drawn under the cover glass as the preparation dries. When solid, Rezyl is not as hard as balsam but it is firm enough for ordinary microscopic preparations. When formaldehyde is included in the mixture, the cytoplasm stains as well as the chromatin. The stain, however, is lighter and need not cause trouble.

AMBEROL 750<sup>2</sup>

Amberol 750 is a clear, transparent, alcohol-soluble resin with an index of refraction of 1.536 and a relatively high melting point. It is insoluble in water and in aromatic and aliphatic hydrocarbons but it is soluble in mixtures of phenol, propionic and acetic acids and water. It is not soluble in the usual aceto-carmines and propionic-carmines fluids but very soluble in those which dissolve Venetian turpentine. It is a light straw color in solution and dries to films which are practically colorless.

The principles involved in incorporating a fat-soluble resin in a fixing and staining mixture have been discussed in some detail (Zirkle, 1940). The formulas for mixtures containing Amberol 750 are essentially like those for Canada balsam, sandarac or Venetian turpentine except that minor changes can be made to take advantage of the much greater tolerance of Amberol 750 for water in the solution. The following proportions are suggested as a starting-point but it is emphasized that these proportions may be altered to advantage to fit particular research problems.

Amberol 750.....	.20 g.
Phenol (crystals).....	35 ml.
Propionic acid.....	20 ml.
Acetic acid.....	5 ml.
Water.....	20 ml.
Fe (NO <sub>3</sub> ) <sub>3</sub> · 9 H <sub>2</sub> O.....	0.4 g.
Carmines.....	To saturation

The above mixture is satisfactory under a variety of conditions. A still greater tolerance for water can be secured, however, if some relatively non-volatile liquid such as "Carbitol" (diethylene glycol monoethyl ether) or dimethoxytetraethyleneglycol<sup>3</sup> be substituted for a part of the Amberol. Thus 15 grams of Amberol plus 5 ml. of Carbitol will dry to form a solid, firm and clear mounting medium even if the mixture contains as much as 25 ml. of water. The extra water may be necessary if the specimen to be fixed is extremely delicate.

Orcein should not be substituted for carmine in this formula. It offers no advantage as the carmine practically never stains the cytoplasm but only the chromatin. Then too, if carmine is used, the resin is practically colorless when it hardens, but with orcein it re-

<sup>2</sup>Amberol is made by the Resinous Products and Chemical Co., Washington Square, Philadelphia, Penna. The writer wishes to thank Mr. Philip B. Taft of this company for samples of Amberol.

<sup>3</sup>These compounds can be purchased from the Carbide and Carbon Chemicals Corporation, 30 E. 42nd St., New York 17, N. Y.

mains heavily stained for some time. The Amberol will go into solution quicker if it is ground in a mortar before it is added to the mixture. It should dissolve completely in 12 hours. The iron mordant should be dissolved before the carmine is included. More carmine should be added than will dissolve. The fluid need not be filtered as the excess carmine will settle to the bottom of the container and the liquid will clear. This fixative should be employed in the same volume as the Rezul solutions. Flaming the slide is neither necessary nor desirable.

*Advantages:* As a mounting medium Amberol 750 has the advantages of the natural balsams. The combined fixing, staining and mounting media which have been described previously could not contain safely more than from 13-14% of the natural balsams. If more were added the preparation would frequently become opaque in drying, but mixtures can be made which contain more than 25% Amberol, and dry to perfectly transparent films. In moist atmospheres, slides made from the balsam mixtures had to be dried on hot plates or even in paraffin ovens, a procedure which is not necessary for the Amberol mounting. No mounts in Amberol have clouded even when they were exposed to moist summer weather while they were drying. When dry, Amberol is hard and adheres firmly to glass. The fixing mixture which dissolves Amberol has many advantages over 45% acetic acid as it preserves the chromatin with a sharper outline and shows more detail.

*Disadvantages:* The chief limitation to the use of Amberol is inherent in the incorporation of a water-insoluble substance in a fluid containing water. It should be stated, however, that the test slides have been made carelessly, with no particular precautions and they have been exposed to rough usage but they have not deteriorated. Possibly under humid tropical conditions the mounts would have to be dried artificially. Sometimes whole uncrushed cells in slides which have been aged by exposure to a large daily fluctuation in temperature lose some of the original swelling but chromosomes thus mounted have kept their characteristic structure.

#### BAKELITE BR-7160<sup>4</sup>

Bakelite BR-7160 is an opalescent, alcohol and fat soluble resin with an index of refraction of 1.652. When dissolved in fixing and staining fluids it makes them cloudy, and seemingly unfit for micro-

<sup>4</sup>A product of Bakelite Corporation, 30 E. 42nd St., New York 17, New York. The writer is indebted to Mr. Preston H. Scott and Dr. H. L. Bender for their courtesy and assistance and for supplying this resin.

scopic preparations. The solution, however, can be clarified by filtering and the filtrate remains completely transparent even when dried and hardened. Bakelite BR-7160 is more tolerant of water than Amberol 750. On the other hand, comparable solutions of Bakelite dissolve less carmine so it is necessary, if the Bakelite preparations are to be stained sufficiently, to alter the proportions of the other ingredients so that more carmine will dissolve. This can be done by increasing the relative amount of water. The following preparations are recommended.

Bakelite BR-7160 . . . . .	20 g.
Carbitol . . . . .	5 ml.
Phenol . . . . .	30 ml.
Propionic Acid . . . . .	20 ml.
Acetic Acid . . . . .	5 ml.
Water . . . . .	20 ml.
Fe(NO <sub>3</sub> ) <sub>3</sub> ·9 H <sub>2</sub> O . . . . .	0.4 g.
Carmine . . . . .	To saturation

The phenol, acids and water should be mixed first and then the ground up Bakelite and the iron salt added. It is best to agitate the fluid so that the Bakelite does not form large clumps. After the iron has dissolved, the carmine should be added. The solution should not be filtered until all of the Bakelite has dissolved and until it has become saturated with carmine. Twenty-four hours is generally long enough to wait before filtering. The fluid should not be heated.

Bakelite is not soluble in aceto-carmine but is soluble in propionic-carmine. The writer has pointed out (1933,1940) the possibility of substituting propionic for acetic acid in the usual cytological fixatives and also some of the advantages of the substitution. Forty-five per cent acetic acid (by volume), the standard amount in aceto-carmine, is approximately 8 molar. An 8 molar concentration of propionic acid would be, in round numbers, 60% (by volume). This strength propionic acid will dissolve enough Bakelite to serve as a practical mounting medium. If a 40% solution of formaldehyde be used in place of the water, the fluid gives a fixation image which shows more nuclear detail. The following proportions have proven satisfactory.

Bakelite BR-7160 . . . . .	25 g.
Carbitol . . . . .	10 ml.
Propionic acid . . . . .	60 ml.
40% Formaldehyde . . . . .	40 ml.
Fe(NO <sub>3</sub> ) <sub>3</sub> ·9 H <sub>2</sub> O . . . . .	0.4 g.
Carmine . . . . .	To saturation

This Bakelite solution can not absorb as much water as the one which contains phenol. However, if the specimen is not large and elementary precautions are observed not to bring extra water on to the slide with the specimen, there should be no trouble. No special precautions are necessary in drying the slides. If any air is drawn under the cover glass as the preparation ages, more Carbitol should be added to the fixing mixture.

*Advantages:* Bakelite has essentially the same advantages as Amberol. In addition its greater tolerance of water allows it to be dissolved in 60% propionic acid and thus it can be incorporated in a propionic-carmin solution, and in a fixing and staining mixture which contains formaldehyde.

*Disadvantages:* Bakelite likewise has the limitations of Amberol. Some of the margin of safety gained by its use is lost through the necessity of increasing the proportion of water in the mixture so that enough carmine will dissolve to stain the specimen adequately. The Bakelite fluids have to be filtered which is not necessary with those which contain Amberol. It is also necessary that a non-volatile liquid such as carbitol be incorporated to prevent the mount from cracking with age. Carbitol is also required in the propionic acid formaldehyde mixture to keep the preparation from clouding as it hardens. While formaldehyde increases the amount of nuclear detail preserved, it fixes the cytoplasm so that it stains with carmine. Orcein, in the Bakelite mixtures gave very erratic results. Films of Bakelite dried on glass and exposed to light and air acquire a brownish tint. This discoloration has not yet been observed to occur under cover glasses but there is a definite possibility that microscopic preparations of Bakelite will be discolored in time. Thus far, the discoloration does not appear to be sufficient to interfere in any way with the visibility of the mounts.

#### LACTO-GELATIN

Gelatin has long been a component of mounting media. When used by itself it becomes too hard and brittle for permanent preparations but when mixed with glycerin and boiled in 45% acetic acid it acquires a satisfactory consistency. The chemical changes induced by the boiling in acid need not concern us here. Unfortunately glycerin and the other non-volatile liquids which give the mounts a proper consistency plasmolyze any cells not broken by the smearing technic unless they are incorporated in the fixing and staining fluids in small mounts. Thus the media cannot contain much of the non-volatile components, 8% of the total volume being the upper usable

limit for glycerin while the glycerin and gelatin together should not exceed 15%. This means that the final volume of the preparation will be less than one sixth of that of the fluid in which the specimen is mounted. Unless the smear is clean and thin, air may be drawn in under the cover slip as the preparation dries. The necessity of any extra precaution limits the applicability of the technic.

Lactic acid is a non-volatile liquid which forms an excellent mounting medium with gelatin — firm, tacky and lasting. Mixed with acetic acid it apparently penetrates more rapidly than glycerin and can be used in much greater concentrations without causing the cells to shrink. Its use was not advised, however, (Zirkle, 1940) for it destroyed the color of aceto-carmin preparations; no matter how well the chromosomes were stained originally, they were colorless after twelve hours in lacto-gelatin.

The introduction of aceto-orcein by La Cour has given this mounting medium a certain value. La Cour has reported that orcein — dissolved in equal parts of glacial acetic acid, lactic acid and water—will fix and stain chromatin, and<sup>4</sup> that such preparations will last for a week or two. The incorporation of gelatin into the mixture will make such mounts permanent. Thus 10 grams of gelatin added to 30 ml. each of acetic acid, lactic acid and water form a usable medium, which, however, contains more lactic acid than is needed. The following proportions are less apt to cause unbroken cells to shrink.

Gelatin...	10 g.
Lactic acid .....	15 ml.
Acetic acid. . . . .	30 ml.
Water....	45 ml.
Orcein <sup>5</sup> .....	To saturation

The gelatin should first be dissolved in the water and the acids and orcein added. The whole should be boiled for two or three minutes. It is not necessary to filter as the undissolved orcein will settle to the bottom of the container. For exceptionally delicate material the mixture can be diluted with ordinary aceto-orcein. Flaming is a necessary part of the fixation.

*Advantages:* Lacto-gelatin is firmer and more tacky than Rezyll 7020 and hardens much faster. As it is soluble in water it can be used under almost any conditions.

*Disadvantages:* As stated, lacto-gelatin cannot be used with carmine. When the mounts are exposed daily to direct sunlight even the orcein fades in about two months. Slides kept away from the

<sup>4</sup>The writer employed a Coleman and Bell orcein which is understood to be a recently imported product (non-synthetic).

light have preserved their color, although it is too soon to state whether or not the stain is permanent.

The following medium is suggested as a temporary substitute for Rezul 7020. As it contains formaldehyde the amount of gelatin must be reduced for, even in the presence of the acids, the formaldehyde will cause the gelatin to solidify if more than the indicated quantity is used.

Gelatin.....	5 g.
Lactic Acid.....	30 ml.
Acetic Acid.....	30 ml.
40% Formaldehyde..	70 ml.
Orcein.....	1 g.

The gelatin and orcein should be dissolved in the acids. Heating the mixture is advised. The formaldehyde should not be added until it has cooled.

*Advantages:* This mixture gives the fixation image of acetic acid formaldehyde.

*Disadvantages:* The medium is not as firm as the lacto-gelatin preparation which contains more gelatin and less lactic acid. If the amount of lactic acid is reduced crystals will form as the mount hardens. The staining has thus far proven to be erratic.

#### GENERAL CONSIDERATIONS

The advantages of a single fluid which fixes, stains and serves as a mounting medium are obvious. The chief value of such a one-process technic would be lost, however, if it were necessary to employ it with great care or to take extra precautions. Thus the greater its margin of safety the easier and more widespread should be its use. The four mounting media described here are, within their listed limitations, a marked improvement over those described earlier (Zirkle, 1937, 1940).

How permanent the preparations are it is impossible to say. Slides have been exposed for over a year without protection in a south window, where they are subjected to periodic direct sunlight, and have not deteriorated. (Except that the lacto-gelatin-orcein slides have faded.)

The cytoplasm stains with carmine in those preparations which contain formaldehyde but not in those which contain phenol. Both types of preparations show more nuclear detail than is preserved by 45% acetic acid.

Many of the promising newer synthetic resins have not been tested. There are probably many others now available which are just as useful as those described, and newer ones will probably be even more

serviceable. It seems to be only a question of time until some resin is found which will have all the advantages and none of the limitations of those described here.

The writer wishes to thank Dr. John G. Miller for measuring the indices of refraction of Amberol 750 and Bakelite BR-7160.

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## ELIMINATION OF DISTORTION AND POOR STAINING IN PARAFFIN SECTIONS OF PLANT TISSUES

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**ABSTRACT.**—Three fixing solutions causing least distortion and bright staining of plant tissues are named. Glycerin dehydration causes less distortion than a series of alcohol concentrations; 95% alcohol removes some of the glycerin, sets the protoplasm and improves the staining. Absolute alcohol causes distortion and should be avoided. Pure chloroform, as a paraffin solvent, is followed by brighter staining but more distortion than are the butyl alcohols. A schedule resulting in minimum distortion is given. The results are shown in photomicrographs. Brightest staining follows the use of C. P. iron alum and hematoxylin. The use of a paper cup for very gradual change from one liquid to another and as a labor saver is described.

After conducting numerous experiments over an extended period of time we have obtained considerable evidence regarding factors responsible for distortion and poor staining in paraffin sections of plant tissues. We are offering our results at this time with the hope that they may be helpful to others working in this field.

The first essential for good results is, of course, a good fixing solution. Of the many solutions described in the literature we find Karpechenko's solution<sup>1</sup> to cause the least distortion in the largest number of plants. If differential staining is not sufficiently bright when this solution is used, Flemming's strong (half strength)<sup>1</sup> or Allen's B17 should be tried. The latter two solutions are especially good for fungi. Fixation should ordinarily be for around 48 hours. Since Allen's B17 is not readily found in the literature we are giving its composition below. It should be made up immediately before use, the ingredients being added in the order named.

Saturated solution of picric acid. . . . .	75 ml.
Formalin. . . . .	25 ml.
Acetic acid. . . . .	10 ml.
Chromic acid . . . . .	1.5 g.
Urea. . . . .	2 g.

After washing the tissues 6 to 12 hours in running water we come to the problem of dehydration. Many experiments have convinced us that dehydration by means of glycerin causes distinctly less distortion than the long series of ethyl alcohol concentrations commonly used. The glycerin solution is made as follows: Make a 10% aqueous

<sup>1</sup>See Rawlins, T. E. 1933. *Phytopathological and Botanical Research Methods*. John Wiley and Sons, Inc., New York, N. Y.

solution of glycerin. Add 1 ml. of a 10% solution of thymol in 95% alcohol to each 100 ml. of the glycerin solution to prevent growth of fungi. Fill an evaporating dish with about 100 ml. of this glycerin solution and leave the tissues in the solution until the water has evaporated. This requires about 13 days if the evaporating dish is kept on top of the paraffin oven. A canopy of stiff cardboard or other material should be suspended about 6 inches above the evaporating dish to decrease the amount of dust falling into the evaporating dish. If the blocks of tissue tend to settle on the sides of the evaporating dish during evaporation, push them to the middle of the dish so that they will remain covered by the concentrating glycerin solution.

After all of the water has evaporated the tissues are run through several changes of 95% ethyl alcohol. This treatment removes some of the glycerin, sets the tissues and improves their staining qualities.

The use of absolute ethyl alcohol should be avoided if one desires minimum distortion of tissues. If distortion is of less importance and best differential staining of nuclear structures is a primary consideration, absolute alcohol should follow the 95% alcohol and the absolute alcohol should be followed by a good fat and paraffin solvent such as pure (anesthetic grade) chloroform. Equal parts of absolute ethyl alcohol and 95% alcohol give a mixture that is miscible with chloroform. It is possible that this alcohol mixture may cause less distortion than absolute alcohol. More experiments are needed before we can recommend this mixture. If minimum distortion of tissues and moderately bright staining are desired, we have found 95% ethyl alcohol followed by gradual infiltration with normal butyl alcohol to give best results.

In going from ethyl alcohol to a paraffin solvent best results are obtained if a gradual change is produced by the diffusion of liquids through an onion skin paper cup. This paper is widely used in typing and is therefore readily available. The diffusion method saves much time ordinarily spent in changing a long series of solutions.

The procedure is as follows: Cut the onion skin paper into squares 4 to 6 inches across. Place the basal end of a test tube in the center of a square of paper and press the paper tightly (without moistening) around the test tube to produce a paper cup. (The creases in the paper are made tighter if the test tube having the lower end covered by paper is forced into a vial which is slightly larger than the test tube and is then rolled around to tighten the creases in the paper.) Remove the paper cup and cut off the upper portion with scissors to produce a cup about an inch deep. Add about 17 ml. of paraffin solvent to a test tube having an inside diameter about equal to that of the vial. Place the paper cup in the top of the test tube and place 1 or 2 ml. of the desired strength of ethyl alcohol in the cup by means of a pipette

or medicine dropper. Push the paper cup down until the liquid levels outside and inside the cup are about the same. Place the blocks of tissue in the cup by means of a wire loop such as is used by bacteriologists. (Bend the wire so that the loop is horizontal when the wire is vertical.) Cork *tightly* with a rubber stopper and allow diffusion to go until the alcohol around the tissues has been replaced by the paraffin solvent. (Do not allow the stopper to touch any liquid.)

A similar paper cup technic is used to obtain gradual infiltration of paraffin into the tissues. However in this case a vial about 2 inches deep is used. Here the tissues are in the bottom of the vial in the paraffin solvent and the melted paraffin is poured into the paper cup.

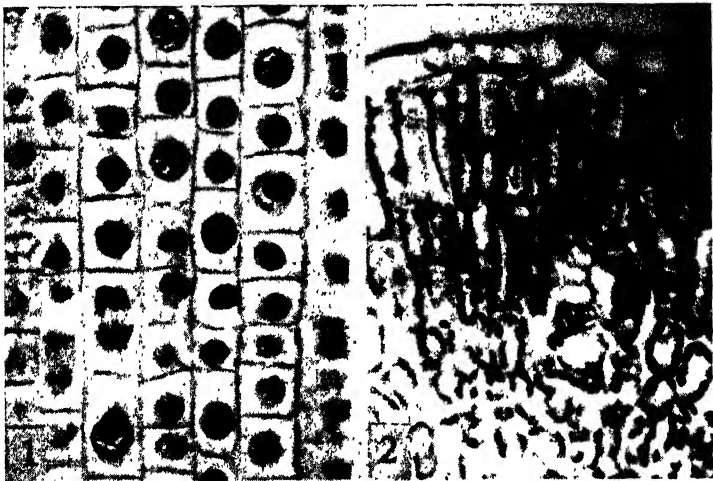


FIG. 1.—Longitudinal section of onion root tip.

FIG. 2.—Cross section of leaf of *Malva parviflora*. (Both sections were prepared by means of the glycerin-95%-alcohol-butyl alcohol combination and were stained with Heidenhain's iron-alum-hematoxylin.)

The paper cup technic should be of wide application in the gradual change from any liquid to another and would save cytologists much unnecessary work in changing long series of solutions.

The following detailed schedule has been more satisfactory for the largest number of plant species than any we have tried:

1. Fix tissues about 48 hours.
2. Wash 6 to 12 hours in running water.
3. Dehydrate the tissues with glycerin.
4. By means of a wire loop transfer tissues to 95% alcohol in a vial. Cork the vial and leave for 2 hours.
5. Give the tissues a second treatment of 1 hour in new 95% alcohol.

6. Place tissues in 1 to 2 ml. of 95% alcohol in an onion skin paper cup suspended in a test tube containing 17 or more ml. of normal butyl alcohol. Stopper the test tube *tightly* and allow diffusion to proceed for 24 hours.
7. Place the test tube on top of the paraffin oven for 2 days to complete the diffusion.
8. Subject the tissues to 2 treatments of new normal butyl alcohol of 24 hours each and 2 treatments of 48 hours each. The vials should be kept on the paraffin oven to hasten the removal of glycerin. Keep the vials corked.
9. Place about 2 ml. of melted paraffin in an onion skin paper cup and suspend this in about 10 ml. of normal butyl alcohol containing the tissues. Place the vial in the paraffin oven for 24 hr. The vial may be covered by a loose cork or small piece of paper.
10. Remove the cork or paper cover and leave the open vial in the oven until the paraffin has reached a constant volume. Remove the paper cup from the vial as soon as it is empty. About 6 days are usually required between the time of removal of the cup and the attainment of constant volume.
11. Pour off the melted paraffin and subject the tissues to 2 one-day treatments and 1 two-day treatment in new melted paraffin.
12. Embed the tissues in the usual manner. (If lignified tissues are too hard to section, the usual softening of the embedded tissues for several days in water or 9 parts of 60% alcohol and 1 part of glycerin should be carried out.)

The absence of distortion or shrinkage of the protoplast after running up through this schedule is shown in figures 1 and 2. In the meristematic region of the onion root there is essentially no shrinkage of the protoplast from the wall. Even in the mature *Malva* leaf the chloroplasts have remained in essentially the same position they occupied in the living cells.

For brightest staining with Heidenhain's iron-alum-hematoxylin the purest grades of iron alum and hematoxylin should be used. We have had best results with "Baker's Analyzed" C. P. ferric ammonium sulfate,  $\text{Fe}_2(\text{SO}_4)_3(\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ . Only the purple portion of the crystals should be used and the solution should be filtered before using. The hematoxylin should also be of the C.P. grade. The proper sequence of distilled water and tap water<sup>2</sup> is also necessary for best results with this stain.

If one does not desire to wait several months for a hematoxylin solution to ripen, the ripening may be accomplished by drawing air through the solution for 2 or more hours.

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<sup>2</sup>See footnote 1.

## GALLOCYANIN AS A NUCLEAR STAIN

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**ABSTRACT.**—Gallocyanin has been used successfully as a nuclear stain. Sections are cut by the freezing method of either fixed or unfixed tissue. The tissues are warmed (not exceeding 70°C.) for 2–4 minutes in the gallocyanin solution. A counterstain may be used if desired. The most effective are Biebrich scarlet, phloxine, or eosin Y. The sections are then dehydrated and mounted in clarite. The nuclear pattern is clearly demonstrated and the sections are permanent.

### INTRODUCTION

Gallocyanin (C.I. No. 883) has been used as a stain by previous workers both singly and in combination. It has been described as a nuclear stain and as a specific stain for the Nissl substance of the motor cells of the central nervous system. Proescher and Arkush (1928) found that the iron lake of gallocyanin was a good nuclear stain and was adequate when substituted for hematoxylin. It has also been used as a constituent of Foley's (1943) combined method for the staining of nerve fibers<sup>1</sup>. Einarson (1932) has used gallocyanin for the demonstration of the Nissl substance. Bruzaglo (1934) used it in combination with orcein and alizarin blue as a tissue stain.

Although hematoxylin is effective in demonstrating the nuclear pattern, it is somewhat difficult to use and does not always give consistent results. It would be advantageous to have a stain for use in normal and pathological histology that would yield consistently a clear nuclear pattern by a simple quick method. In an attempt to supply this need, this paper is concerned with a description of the use of gallocyanin in the staining of frozen sections by the spot plate method of Cole (1947).

### MATERIALS AND METHODS

#### STAINS

It was found that the iron lake of gallocyanin, as described by Proescher and Arkush (1928) washed out of the tissues when used with the spot plate method; even if it were successful the procedure is slow.

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<sup>1</sup>Foley (1943) describes alizarin blue in his method for staining nerve fibers. Conn (1946) classifies this as gallocyanin.

Several trials indicated that modification of Einarson's formula (1932) was best for rapid staining. The following formula was used:

Gallocyanin.....	1.5 g.
Chrome alum.....	5.0 g.
Distilled water.....	100 ml.

Mix the chrome alum ( $K_2SO_4 \cdot Cr_2SO_4 \cdot 24H_2O$ ) and the water. Heat to boiling. Add the gallocyanin and boil for about 5 minutes. Filter while hot and store in a dark bottle.

It was found that the stain is rather unstable in solution<sup>2</sup> and should be prepared freshly every two weeks. The dye has a tendency to precipitate out of solution and hence the stain loses its characteristic qualities.

#### METHODS

Tissues prepared by the frozen section method from 5 to 15  $\mu$  in thickness, were put successively in a shallow dish containing 0.9% sodium chloride solution, next in a shallow dish containing 25 ml. of the stain in which they were warmed gently (not more than 70°C.) for 1 to 3 minutes, depending upon the size and type of tissue. Staining can be done in a cold solution but it requires 5-8 hours. The intensity of the stain may be ascertained by removing a section and examining it microscopically. When the staining has progressed sufficiently, the sections are washed in distilled water and dehydrated. Counterstaining is not necessary, if nuclear patterns alone are being studied, but for some tissues it may be desirable to use a counterstain in order to demonstrate the cytoplasm. The sections may be cleared in any of the common clearing solutions. In this laboratory good clearing is obtained by using beechwood cresote (Merck) or this solution:

Xylene.....	5 parts
Toluene.....	1 part
Beechwood cresote.....	1 part
Aniline oil.....	1 part

In general the following procedures have given the most satisfactory results. The alcohol used was methyl alcohol.

1. Gallocyanin as described.
2. Distilled water, 2 minutes before Biebrich scarlet, (C.I. No. 280) or 15 seconds before phloxine (C.I. No. 778, certification No. NPh 7)
3. Alcohol, 25%; 15 seconds
4. Alcohol, 50%; 15 seconds

<sup>2</sup>This subject is under investigation and it is hoped to alter the stain so as to increase its stability without losing any of its advantages.



FIG. 1.

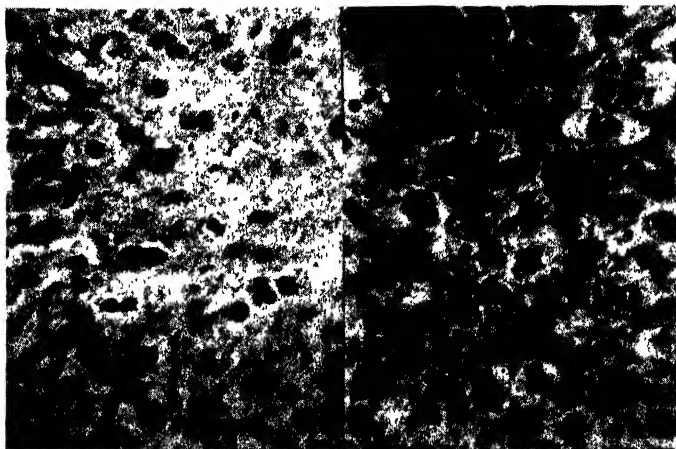


FIG. 2.

Biospy of neoplasm removed from the distal third of the finger over the base of the nail. Photomicrographs demonstrate nuclear pattern stained by gallocyanin with no counterstain. Frozen sections prepared from tissue fixed in 10% formalin for 24 hours. Fig. 1 Magnification  $\times 120$ , Fig. 2 magnification  $\times 576$ .

5. Biebrich scarlet, 1%, 30 seconds, or 0.25% phloxine, 15 seconds (both in 75% methyl alcohol)
6. Alcohol, 90%; 15 seconds
7. Clear, 1 minute
8. Mount in clarite

Eosin Y has also been used and has given best results with a 0.25% solution in 75% methyl alcohol. Overstaining occurs easily with this stain. In this laboratory best results have been obtained by using sections no thicker than 15  $\mu$ . Sections cut at 20–30  $\mu$ , used for biopsy evaluation, are not too thick for satisfactory results with this stain.

### DISCUSSION

Either fresh frozen sections from biopsy or fixed sections cut by the freezing method can be treated by this method. Type of fixation does not seem to affect results, and in cases where a fine differentiation is required and not obtainable with hematoxylin this stain can be used with advantage. Since over 1000 sections have not visibly deteriorated in twelve months, it can be assumed that this stain is relatively permanent.

Gallocyanin without a counterstain gives a clear nuclear picture with a faint blue background. In some tissues, such as striated muscle, the faint blue background is sufficient for a detailed study.

Malignancies stain well, cellular infiltrations are well differentiated and the nuclear pattern is well shown.

Although the method is not as rapid as the spot plate method employing hematoxylin and eosin Y (Cole, 1947), nuclear detail is better. It is not as well adapted to quick evaluation of biopsy material as the former method but for fine differentiation of cellular elements it seems to be more valuable. Time of staining employing the hematoxylin and eosin Y need not exceed 2 to 3 minutes, while in using gallocyanin 10 minutes may be required.

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## STAINING OF EMBRYONIC AND SMALL MAMMALIAN SKELETAL SYSTEMS

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The methods commonly used for staining developing bone (Lundvall, 1905; Spalteholz, 1914; Batson, 1921; Dawson, 1926; Richmond and Bennett, 1938; Cumley, Crow, and Griffin, 1939; Williams, 1941), have involved the use of 1% or 2% aqueous KOH without employing any bleaching agent. The introduction of hydrogen peroxide into the technic proves to be a distinct advantage, and in the concentration here proposed, does not result in the production of fine bubbles. The maceration process can be hastened through the use of an increased concentration of potash in the absence of a fixative, although this in itself is one of the hazards of the technic. However, if the preparations are carefully watched, this difficulty may be largely avoided. The method works admirably and gives a very clear muscle tissue so that the skeletal elements are very sharply outlined. The proposed method (arranged in the form adopted in Staining Procedures) is herewith described.

Specially recommended for: Vertebrate embryos and small adult vertebrates.

Designed to bring out: Developing bone.

Method of fixation: omitted

Preparation of staining solution: Alizarin red S, (C C.), 0.001 g.; 2% aqueous KOH, 100 ml.

Staining schedule:

1. Place fresh specimens in 5-10% aqueous KOH, to which has been added a few drops of 2% hydrogen peroxide, until the bones are clearly visible through the surrounding tissues.  
Note: If close supervision is impracticable, the specimen may be placed in 2% aqueous KOH (e.g. overnight) and then replaced in the more concentrated potash solution. If the tissues become too soft, harden 12-24 hours in a solution of equal parts glycerin, 95% alcohol, and water, and return to the clearing process.
2. Transfer to dilute (0.0025-0.01%) alizarin red S in 2% aqueous KOH until the desired degree of staining is obtained. The percentage solution is proportional to the size of the animal being stained and is greater in adult specimens.

Note: The use of acid alcohol in the case of overstaining is strongly advised against since the acid solution brings about the decalcification of minute ossification centers. If the dilution of alizarin is maintained at the proper level, overstaining will not occur.

3. Glycerin series: (72 hours)

25 parts glycerin, 75 parts 2% aqueous KOH

50 parts glycerin, 50 parts 2% aqueous KOH

75 parts glycerin, 25 parts 2% aqueous KOH

100% glycerin

4. Store in glycerin (CP) to which a crystal of thymol has been added to prevent mold.

Results: Osseous tissue—red

Soft tissues—transparent and unstained

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## SIMPLIFIED PERMANENT ACETO-CARMINE SMEARS WITH A WATER-MISCIBLE MOUNTANT

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**ABSTRACT.**—By employing a water-miscible mountant with a good refractive index, permanent aceto-carmine smears can be made from any of a variety of killing, fixing, and staining schedules. Clearcol, a commercial preparation, originally developed for mounting fungi from various water-containing media, is employed for anther smears by placing the medium directly on the final stage of aceto-carmine staining schedule. Thus, no dehydration and clearing are necessary. By controlling amounts of acetic acid either mixed with Clearcol or on smears, clear, well-stained preparations can be produced.

Ever since Belling's (1926) original work with iron-aceto-carmine preparations of anther smears for chromosome studies, there have been developed various technics for making such material permanent. McClintock's (1929) method, employing balsam or related mountants has had wide acceptance. Various mixtures of gum, resins, and other substances soluble in water, alcohols, hydrocarbons, or acids have been tried by Zirkle (1940) to reduce killing, fixing, staining, and mounting to one operation. The limitations of these combination methods are recognized by him.

The method proposed here employs a commercial preparation, Clearcol, which is miscible in water and acetic acid. It is a medium consisting of a mixture of plastics and solvents which, on evaporation of the volatile constituents, results in a clear non-crystalline preparation. According to information furnished by the originators of Clearcol, (H. W. Clark, the manufacturer<sup>1</sup>, of Washington, D. C., and W. W. Diehl, of the U.S. Department of Agriculture), it was developed primarily for making permanent mounts of fungi without the necessity of dehydration. After the desired staining reaction has been secured from any of a number of killing, fixing, and staining schedules, the cover glass is removed (small 1/2" or 5/8" circles are excellent) and the Clearcol placed on the cover glass, care being taken to avoid air bubbles. The slide is then inverted over the cover glass

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<sup>1</sup>Clearcol may be purchased on application to Mr. H. Willard Clark, 5419 32nd St., N.W., Washington 15, D.C.

and carefully pressed against the Clearcol, the whole brought right side up and the cover glass allowed to settle of its own weight. If more pressure is desired to flatten the cells, this may be applied after the cover glass has settled. In the event that the cover glass and slide are to be mounted separately to avoid different levels of focusing, a clean slide for the cover glass containing the smeared cells, and a clean cover glass for the slide with its cells may be employed.

The mountant, Clearcol, has considerable permanence. Fungi mounted with this medium have remained in an excellent state for as long as eighteen years in the mycological collections of the Bureau of Plant Industry. If any cracking of the dried Clearcol shows up near the edge of the cover glass, this can be remedied by placing a small amount of glycerin around the edge. This will be slowly absorbed by the Clearcol and retained. The refractive index is very close to balsam. It does not affect phloxine adversely after 18 years, nor does it appear to have any ill effect on carmine at the end of one year.

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## STAINING NERVE FIBERS WITH METHYLENE BLUE. AN EVALUATION OF VARIABLES USED IN AN IMMERSION TECHNIC

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**ABSTRACT.**—A study of the effects of osmotic pressure, pH, the presence of dextrose, acetate, pyruvate or lactate, and agents affecting cell permeability during supravital staining by methylene blue was made by means of an immersion technic. Mesentery and intestine of dogs and cats were used. Penetration of the dye was limited to the mesentery and more superficial layers of intestine. Conditions which facilitated the characteristic differentiation of nerve fibers were: continuous oxygenation of the staining solution, pH about 5.6 stabilized by phosphate buffer, and the presence of small amounts of acetate and lactate. Young animals' tissue stained better than old. Methylene blue was a much more effective staining agent than less completely methylated thionins.

In the experiments which form the basis of this report, an analysis was made of the factors which influence the staining of nerves supravitaly by methylene blue. A series of tests on the composition of staining solutions and on the effects of variations of temperature and oxygenation were run under carefully controlled conditions. Comparable observations of the type, specificity, and density of staining reactions were made possible by the use of an immersion staining technic of thin tissues and by the utilization of a number of similar specimens from a single animal.

### EXPERIMENTAL PROCEDURE

The following factors were included in the studies made on the composition of the staining solutions: 1) osmotic pressure; 2) pH; 3) presence of dextrose, acetate, pyruvate or lactate; and 4) agents affecting cell permeability. In addition several experiments were made at different temperatures and different degrees of oxygenation.

Four-inch lengths of small intestine were removed from cat or dog under sodium pentothal anesthesia and immersed immediately into the staining solutions to be tested. Wide-mouthed specimen bottles

<sup>1</sup>Contribution No. 473.

(120 ml.) were used for the immersion fluid and each bottle was provided with an 8-mm. glass tube dipping to the bottom and connected to an oxygen tank through a pressure-reducing valve and distribution system to 10 bottles. The mesentery was left attached to each segment of the intestine and provided a thin tissue while the intestinal wall served as a thick one. The progress of staining was observed under a 15-power binocular dissection microscope at time intervals of adequate frequency to note changes in staining.

Nineteen dogs and eight cats were used, and an average of about eight individual staining experiments per animal performed. Representative specimens of the total 230 pieces of intestine used were saved for embedding, sectioning and further observation under higher magnification only when the results as seen with the binocular during staining seemed to justify it. Whole mounts of the mesentery were made occasionally.

Variations of the staining solutions and other variables are discussed under the individual headings which follow.

#### THE SUBSTRATE

(a) *Temperature.* Trials of 26°, 37–38°, and 46°C. showed that the normal mammalian body temperature was the most effective one. Only diffuse staining wherein no nerves appeared was obtained at 46°.

(b) *Oxygenation.* Fresh tissue immersed in the unaerated staining solution gave only moderate selectivity for neural elements. When air or oxygen was bubbled through, selectivity was found to be much increased. Pure oxygen was more effective than air. Although it is best to differentiate tissue that has been perfused with methylene blue in a physiological saline solution without dye, the procedure of immersion first in the dye solution and afterward oxygenation in physiological saline was found to be unnecessary. Presumably, the presence of high concentrations of oxygen permits earlier and more complete oxidative differentiation. The best staining in these trials was obtained when the tissue was left in the methylene blue solution at 37–38° C., and pure oxygen bubbled through during the entire 2 to 4-hour staining period.

(c) *Composition of the staining solution.* Dye samples were dissolved in various salt and buffer mixtures, with and without dextrose, to test the efficacy of the carrier.

One series of tests was made to determine the effect of the tonicity of solutions. Concentrations of sodium chloride ranging from 0.5 to 2.0% were used and it was found that solutions from 0.6 to 1.5% stained with about equal effectiveness. However, solutions that were

slightly hypotonic appeared most satisfactory. It is evident that tonicity is not a critical factor in penetration or specificity of staining.

Dextrose has been advocated in many types of media to maintain metabolic activity, and was used in this study. Tests showed that the addition of 0.17 to 2.0% dextrose was of slight value in improving the quality of staining (four hour period). An addition of 0.2% was arbitrarily chosen as one which did no harm, but possibly some good, and was used subsequently.

Schabadasch (1936) and Hillarp (1946) have reported that the pH of methylene blue solutions is important in the staining reaction. The pH of solutions was altered by the addition of phosphate buffers ( $M/15 Na_2HPO_4$  and  $KH_2PO_4$ ), and checked electrometrically with a glass electrode before and after staining. Solutions ranged in pH from 5.15 to 6.9 at the beginning of staining, and the best staining occurred with solutions of pH 5.6. Five milliliters of buffer were sufficient to hold 95 ml. of staining solution at a fairly constant level (below pH 6) during a three to four hour period.

The addition of small amounts of sodium acetate has been advocated by Hillarp, and in our study it was found to facilitate staining. A series of sodium acetate solutions varying in concentration between 0.02 and 0.1% (with phosphate buffer and 0.6% sodium chloride) was tested and maximal staining found to occur with 0.03% solution.

Further experiments were run to find out whether acetate buffer had the same effect. Solutions of N/10 acetic-acid-sodium-acetate buffer (both with and without phosphate buffer) were made at pH 5.6 and added to dye solutions in varying amounts. With large volumes of buffer the sodium chloride content was reduced to keep the solution isotonic or slightly hypotonic. Acetate buffer was tried in 5, 15, 60, and 100% concentrations; it was found that 5% solution (0.005 *M*) gave the best result, but it was not quite as good as the control which contained a similar concentration of sodium acetate with phosphate buffer.

A similar series of pyruvic and lactic acid buffers were used in which the acids were neutralized to pH 5.6 with N/10 sodium hydroxide. They were mixed with dye solutions in a series of proportions representing 10 to 50% of the total volume. It was observed that only the 50% mixture of buffered lactic acid gave good staining. Acetate buffers in comparable concentration inhibited staining of nerves completely.

In another series, solutions of sodium lactate (0.5 molar) were compared with mixtures of salt, dextrose, and phosphate buffer, some with sodium acetate and others without. Solutions containing both

sodium acetate (0.03%) and sodium lactate produced more satisfactory staining than either alone. Sodium lactate seemed to improve staining of finer nerve fibers. It was found that 4 to 8 ml. of 0.5 molar sodium lactate exerted the greatest beneficial effect.

Attempts were made to alter cell permeability, to note the effect on staining by the addition of potassium, calcium, magnesium, or barium chloride (ranging from 0.03 to 1.4%), urea (0.01 to 0.20%), and glycerin (10%). All inhibited staining.

This group of experiments on the composition of the staining solution has led to the recommendation of the mixture given in the outline of technic at the end of this article.

### DYE REACTIONS

Methylene blue (tetramethyl thionin) was used in 0.01% concentration in all of the experiments except those made with other thionin derivatives. Preliminary tests showed that higher concentrations produced more diffuse staining, and more dilute solutions gave a less clear-cut picture. The U.S.P. Medicinal form of the dye was used (National Aniline, Cert. No. 9.).

As used for immersion staining, methylene blue was found to have two phases in its reaction. Fat cells, smooth muscle, connective tissue, and blood cells always stained first. After about one hour, fine nerve fibers and then heavier ones appeared and darkened progressively until, in the typical reaction, they were more prominent than surrounding tissue. The finer fibers then tended to fade while the larger continued to darken. This apparently was due to a combination of penetration, post-mortem tissue change, and oxidation-reduction phenomena. The cause of the rather indifferent staining of smooth muscle, connective tissue, and fat cells was thought to be due to the presence of small amounts of demethylated methylene blue (dimethyl and trimethyl thionin) in the solution. It has been observed that old methylene blue solutions or fresh solutions oxidized with peroxide do not stain nerve fibers, and to verify the effect of demethylation on staining, a series of experiments was run using the azures and thionin.

Samples of trimethyl thionin (azure B), dimethyl thionin (azure A), monomethyl thionin (azure C), and thionin (Lauth's violet) were used in 0.01% concentration in a buffer at pH 5.6 to observe their reaction with the various tissue elements. Controls of methylene blue were run in each case.

Azures A and B stained nerve fibers with about half the intensity of methylene blue controls, while very faint staining occurred with azure C and thionin. The protoplasm of fat cells and smooth muscle

fibers stained intensely with each dye. The color produced ranged from violet to pink, being darkest with azures A and B and lightest with thionin. Solutions of the azures were different from fresh methylene blue in being redder in tone. Methylene blue solutions which stand for more than three or four days take on this red coloration which is apparently due to conversion of the tetramethyl to the tri-, di-, and monomethyl compounds. Such solutions should not be used in staining of nerve fibers.

Holmes (1928) showed by means of spectrophotometric analyses that commercially prepared methylene blue contains about 10% trimethyl thionin. Our studies suggest that this contaminant may be, at least in part, responsible for the staining of elements other than nerve fibers. Both Holmes, and Lillie (1944) prepared nearly pure methylene blue but neither used the material for supra-vital staining. Work is in progress dealing with the purification of methylene blue in attempt to obtain more specific staining.

#### THE EXPERIMENTAL ANIMAL

Early in the course of this study, it became obvious that the nerve tissue of no two animals reacted identically. As a general rule very young animals have tissue which stains well; adult animals have a moderate ability to stain, and old animals frequently have nerves which stain very poorly or not at all. Our data do not permit an evaluation of the causes of such variations, but the role of methylene blue as a hydrogen acceptor in carbohydrate metabolism should be considered in relation to the nutritional state and the aging process. Nutritional experiments might shed light on the problem.

At this time it can be said that nerve fibers from a freshly killed young cat or dog will nearly always stain well. Staining reactions were graded arbitrarily from 0 (with no nerve staining) to 4 (most complete and best differentiated). Table 1 shows the results obtained.

TABLE 1. COMPARISON OF STAINING REACTIONS OF YOUNG AND OLD ANIMALS.

Grade of stain	0	1	2	3	4
Young dogs	0	2	1	4	7
Old dogs	1	1	2	1	0

Although the series is too small for conclusions, tissue from young animals seems to be preferable to that from old.

If the animal has been dead for over four hours, staining is usually unsatisfactory; though if the tissues are kept on ice one may occa-

sionally obtain good staining as long as ten hours after death. The average time limit, however, is two to three hours.

#### PRESERVATION OF SPECIMENS

(a) *Fixation.* The preservation of the stained tissues involves several problems. Fixation must be rapid to prevent fading, and such that the dye will not dissolve out during dehydration and clearing. Eight per cent ammonium molybdate (a commonly used reagent) produces a fairly insoluble precipitate of methylene blue but is a poor tissue fixative. Poljak (1941) has developed a number of improved fixing fluids, by combining formalin or dichromate and either acetic or trichloroacetic acid with the molybdate. We have used a modification of his molybdate, dichromate formula without acid. One per cent potassium dichromate was found to be compatible with 8% ammonium molybdate for at least 24 hours and gave better fixation than molybdate alone. After 24 hours this mixture may precipitate, but tissues that can be stained by immersion would not require more time than this. After a 24-hour fixation, tissues were washed for a similar length of time in several changes of distilled water.

(b) *Dehydration.* Molybdate-precipitated methylene blue is partly soluble in the usual alcohols used for dehydration. Tertiary butyl alcohol (recommended by Levine, 1939) has only a very slight solvent action and was a satisfactory dehydrating agent. It has the disadvantage of penetrating about half as fast as ethyl alcohol so that dehydration takes longer. Segments of bowel required four to five days treatment before dehydration was complete. Another disadvantage is that it has a melting point of 25.5° C., and must be kept above this temperature to prevent crystallization.

Specimens were transferred from the wash water to full strength tertiary butyl alcohol after the excess water was removed with blotting paper. The alcohol was changed twice during the course of dehydration. Clearing with xylene and embedding in paraffin were handled as with ordinary preparations, or whole mounts in clarite were made.

#### SUMMARY

Many reports have been written about the staining of nerve fibers with methylene blue, and each author has advocated certain refinements in technic which have tended to make the procedure increasingly complex. The impression is gained that each modification will yield constant results and yet, in our hands, this has not been true. This study was initiated to isolate and evaluate as far as possible the factors involved.

The methylene blue reaction is characterized by, first, the absorption of the dye with concomitant reduction to the leuco-base and, second, the oxidation of the colorless form of the dye to produce the stain. Whether the dye is reduced to the colorless form depends on the oxygen tension or reducing capacity of a given tissue. During staining by immersion, the superficial structures stain first, since they are in more intimate contact with both dye and oxygen than deeper ones. The first phase of staining is not visibly specific for nerve fibers, but it seems probable that they are concentrating the dye and simultaneously converting it to the colorless reduced form. Later, as their vital processes lag, the oxidation of the dye reveals them more intensely stained than the surrounding tissues. With immersion staining, differentiation of nerve fibers progresses in the presence of the dye, and it is not necessary to differentiate in a solution which contains no dye as has been recommended for perfused specimens. Differentiation appears to depend upon the physiological state of the tissue and the presence of oxygen.

The physiological state of the nervous system is so variable that no two animals react alike to methylene blue as used in present methods. This makes exact repetition of experiments difficult or even impossible. In spite of this, one occasionally obtains very beautiful preparations which clearly demonstrate the architecture. If young, well nourished healthy animals are used, the results are more satisfactory than with older animals.

Important technical factors in immersion staining appear to be the use of the following:

1. Fresh methylene blue solutions.
2. Temperature 37–38° C. (for mammals).
3. Solutions kept saturated with oxygen.
4. pH between 5 and 6, preferably 5.6.
5. Isotonic or slightly hypotonic solutions.

*Suggested outline of immersion technic.*

1. Young, healthy mammals are killed with pentobarbital.
2. Thin tissues are immersed in:

Methylene blue (U.S.P. medicinal) . . . . .	0.01 g.
Sodium chloride . . . . .	0.60
Dextrose, C.P. . . . .	0.20
Sodium acetate . . . . .	0.03
Distilled water . . . . .	90.00 ml.
Phosphate buffer, M/15, at pH 5.6 . . . . .	5.00
Sodium lactate, M/2 . . . . .	5.00

Add the buffer and lactate after the methylene blue has dissolved.

3. Keep the staining solution at 37–38° C. and allow a small stream of pure oxygen to bubble through it during the course of staining.
4. Observe the specimens at 0.5-hr. intervals with 10 × or higher magnification.
5. When the differentiation of nerve fibers appears to be optimum (2–4 hr.) fix in:

Ammonium molybdate . . . . .	8.0 g.
Potassium dichromate . . . . .	1.0
Distilled water . . . . .	100.0 ml.

Renew the solution after 24 hr. if longer fixation is desired.

6. Wash with several changes of distilled water for 24 hr.
7. Dehydrate in pure tertiary butyl alcohol (at 26–28° C.) for 3 to 5 days and change the alcohol twice.
8. Clear in zylene. Prepare whole mounts with clarite or embed in paraffin and section.

Expenses incurred in this research were covered by a grant from the Biological Stain Commission.

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## LABORATORY HINTS FROM THE LITERATURE

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A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS  
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

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### MICROSCOPE AND OTHER APPARATUS

BARRON, A. L. E. **Microscope mirrors.** *Microscope*, 6, 113-4. 1947.

Suggestions for improvements in microscope lighting are made. To achieve a single reflection of the light source, reflection from a first surface mirror made of aluminum vacuum-deposited on glass is recommended. Correct alignment in which the center of the mirror coincides with the optic axis also greatly improves lighting and is easy to attain.—C. Randall.

COLLINS, W. G. **Process timer.** *Microscope*, 6, 115. 1947.

The author describes a timer which can be set for any internal up to 35 min. with an accuracy of plus or minus 2 sec. The use of a small synchronous motor, such as those used to drive industrial recording instruments, is the basis of the design.—C. Randall.

KESSEL, ALBERT M. **Multiple tray for embedding tissues in paraffin.** *J. Nat. Cancer Inst.*, 5, 257-9. 1945.

The author describes an excellent device for mass embedding, consisting of a multiple paraffin embedding tray which has 5 units each containing 10 compartments. Each compartment holds 2 or 3 paraffin blocks depending on the size of the tissues and is separated from its neighbor by easily removable metal strips. The metal parts drop off when lightly tapped after cooling. A complete description of the construction is given.—Paul G. Roope.

SCOTT, T. L. **Slide Rack.** *Microscope*, 6, 122-4. 1947.

Details are given for the construction of a rack measuring approximately  $4 \times 3\frac{1}{4} \times 2\frac{1}{4}$  in. Essentially it consists of 2 plates held apart by threaded rods. The plates are provided with arms which support the slides. Two other slide rack designs are also given.—C. Randall.

### MICROTECHNIC IN GENERAL

KUPPERMAN, H. S., and NOBACK, C. R. **A rapid iron hematoxylin stain utilizing a combined fixative-mordant solution.** *Arch. Path.*, 40, 78. 1945.

The addition of 1% ferric ammonium sulfate to Bouin's solution, (U. S. P. formalin 1:10 containing 0.5% acetic acid) and Lavdowsky's mixture (U. S. P. formaldehyde, 10 ml., 95% ethanol, 50 ml., glacial acetic acid, 2 ml., and distilled water, 40 ml.) gave a combined fixing-mordanting solution which is recommended for decreasing the time required for the Heidenhain staining procedure.

Tissues are fixed and mordanted in any of the above solutions for 1-3 days, depending on their size. They are then dehydrated in ethanol or dioxane for paraffin infiltration, and tissue sections after deparaffination are stained directly in Heidenhain's hematoxylin solution, destained with saturated aqueous picric acid, then counterstained, dehydrated, cleared and mounted in clarite or balsam.—L. Farber.

STEINHERZ, A. R. **The shape of particles in finely ground powders.** *J. Soc. Chem. Ind.*, 65, 314-20. 1946.

Three methods for determining the particle size in the sub-sieve range are discussed. For microscopic counting and measurements, suspensions are pre-

pared of dry cement and glass particles in ethylene glycol, containing 1 mg. sodium citrate per ml. as an anticoagulating agent. Particle counting is carried out with a Thoma counting chamber and an eyepiece micrometer-net.—*R. T. Whittenberger.*

### DYES AND THEIR BIOLOGICAL USES

**CORRIN, M. L., and HARKINS, W. D.** Determination of the critical concentration for micelle formation in solutions of colloidal electrolytes by the spectral change of a dye. *J. Amer. Chem. Soc.*, **69**, 879-88. 1947.

The suitability of a dye as an indicator in the determination of the critical concentration for the formation of micelles in soap solutions is related to the existence of an equilibrium mixture of the dye in aqueous solution from which one form is preferentially solubilized by the soap micelle. The charge on the dye ion must be opposite to that of the micelle. In general, the variation of the spectrum of a dye with change in environment is not a sufficient condition for the suitability of the dye as a critical concentration indicator. Pinacyanol chloride and rhodamine 6 G are shown to be suitable for the determination of the critical concentrations of anionic soaps, while sky blue FF, eosin, fluorescein, and acidified 2,6-dichlorophenolindophenol may be used with cationic detergents. The necessary presence of acids, bases or buffering salts renders the use of acid-base dyes less satisfactory than dyes in which a polymerization equilibrium exists. The critical concentration may be determined by a titration in which the disappearance of micelles is determined by a sharp change in color or by a sharp reduction of fluorescence. Certain dyes exhibit both of these changes.—*R. T. Whittenberger.*

**FISCHER, E., and MUNOZ, R.** Comparative bacteriostatic assays with rosaniline and its phenol analog (rosolic acid). *J. Bact.*, **53**, 381-8. 1947.

Rosaniline and rosolic acid are bacteriostatic at fairly high concentrations. The rosaniline is more effective in alkaline medium than in neutral or acid; the rosolic acid is equally effective at the various pH values tested. Although the lot of rosaniline used was not specified, the rosolic acid was prepared from the same sample of dye.—*V. Kavanagh.*

**TILLEARD, D. L., and SMITH, N. D. P.** Comparison of optical and electron microscopy of pigments. *J. Soc. Chem. Ind.*, **65**, 261-4. 1946.

Optical and electron micrographs of identical pigments are shown. In the electron micrograph a red earth pigment is seen to contain many fine, almost transparent particles which by reason of their small size are not visible in the optical micrograph. In the electron micrograph of zinc oxide, particles are resolved whose shape is undisclosed in the optical micrograph. An important advantage of the electron microscope over the light microscope is that a great depth of focus is obtainable at high magnifications. Caution must be taken in obtaining quantitative data on particle size distribution with the electron microscope, since the field of view is small and large particles may be missed.—*R. T. Whittenberger.*

### ANIMAL MICROTECHNIC

**BAILLIF, RALPH N., and KIMBROUGH, CECIL.** Studies on leucocyte granules after staining with Sudan black and May Grünwald Giemsa. *J. Lab. & Clin. Med.*, **32**, 155-66. 1947.

Dried blood films can be advantageously studied after staining as follows: Fix for 5 sec. in a mixture of 1 volume of formalin and 9 of 95% alcohol, wash in distilled water and dry. Stain with Sudan black B (70% alcohol saturated with the dye) for 30-60 min. at room temperature. (The colors will be darker at 37°C.). Flood the slide with May-Grünwald mixture for 3 min. and cover with neutral distilled water for 1 min. Drain without washing and cover with dilute Giemsa mixture (1 drop stock solution to 2 ml. of water), for 15 min. Wash in distilled water and dry. Erythrocytes are stained slate gray to black. Neutrophile granules are small spherical bodies collected in groups in the cytoplasm. Eosinophilic granules are larger and composed of a peripheral deeply sudanophile shell with a central sudanophobic core. Basophilic granules and azurophilic elements are sudanophobic. Oxidase and peroxidase granules are probably always sudanophilic.—*John T. Myers.*

**BHATTACHARJI, L. M., SINGH, JASWANT, and SENGUPTA, G. P.** A simple methylene blue-eosin substitute for Leishman and Giemsa stains. *Indian Med. Gaz.*, 81, 400-1. 1946.

War shortages necessitated finding a substitute for Leishman and Giemsa stains. After experimenting with different concentrations of dyes and oxidizing agents, the results achieved with the following formula were found to be as good as with any of the standard stains. Preparation of stain: Dissolve 1.0 g. methylene blue in 100 ml.  $H_2O$  and 0.3 g.  $KMnO_4$  in 100 ml. water. (Water used need not be distilled, for slightly alkaline tap water with a pH value of 7.2 to 7.6 is satisfactory.) Transfer the methylene blue solution to a procelain dish and heat so it steams but does not boil for 5 min. Gradually add the  $KMnO_4$  solution. (A fine layer of crystals form on the surface.) Continue heating for 10 min. Dissolve 0.4 g. eosin in 50 ml. water and add to steaming mixture, stirring with a glass rod, while a thick yellow scum appears. Continue heating, not too strongly, for an hour or until the solution has evaporated, leaving a thick scum with metallic luster, at first copper red and when dry, green. (The residue should be left overnight in a desiccator or incubator at 37°C. When completely dry the scum will peel off as shining green flakes. If necessary remove with a scalpel.) Powder the flakes in a dry glass mortar and store in a dry air-tight glass container. As a substitute for Leishman stain put 0.1 g. of the powder in a glass mortar and dissolve thoroughly by grinding it while adding 40 ml. methyl alcohol in small quantities at a time. Transfer to a small bottle and use for staining blood smears, both thick and thin, by following Leishman's method. As a substitute for Giemsa stain, put 0.3 g. of powder in a glass mortar and grind it while very gradually adding a mixture of glycerin and methyl alcohol (25 ml. each). Transfer this solution to a small glass bottle and keep at room temperature until the next day, then place bottle neck deep in a  $H_2O$  bath for 2 hr. Follow Giemsa's method for staining.—G. N. Ludwig. (Courtesy *Biological Abstracts*).

**DEANE, HELEN W., NESBETT, FRANCES B., and HASTINGS, A. BAIRD.** Improved fixation for histological demonstration of glycogen and comparison with chemical determination in the liver. *Proc. Soc. Exp. Biol. & Med.*, 63, 401-6. 1946.

Rossmann's fixative (neutral formalin, 1 part; picric acid, saturated solution in absolute alcohol, 9 parts.) used at 0°C. preserved glycogen in rat liver uniformly throughout the block. The low temperature of fixation gave sections which were uniformly stained, without the usual rim of more intensely stained cells seen after fixation at room temperature. Glycogen granules were distributed throughout the cytoplasm of liver cells rather than concentrated against one membrane. Quantitative determinations of glycogen were made by chemical means and by photometric measurements on the stained histological sections. Comparisons of values obtained showed excellent agreement. Glycogen was stained by the Bauer-Feulgen reagent (See Bensley, C. M., *Stain Techn.*, 14, 47, 1939). Four photomicrographs show the optical density and other characteristics of the stain.—H. A. Davenport.

**DISCOMBE, G.** The nature of neutrophilic granulation. *J. Path. & Bact.*, 58, 572-3. 1946.

As recognized earlier by Lison, the granules of neutrophils and eosinophils are probably lipid. They stain with Sudan black in a manner indistinguishable from that seen in good oxidase preparations. The reason for their staining with Leishman's stain is that one of the components of the stain is adsorbed on the surface of the lipid granule. This component is probably Bernthsen's violet since a simple aqueous solution of this basic dye stains granules well.—S. H. Hutner.

**DIGGS, L. W. and SURRIDGE, MARJORIE S.** A study of the sternal marrow and peripheral blood of fifty-five patients with plasma cell myeloma. *J. Lab. & Clin. Med.*, 32, 167-77. 1947.

Films from sternal marrow stained with Wright stain showed increased cellularity; relative and absolute increase in plasma cells of 4 to 90% (more than 10% in most cases), increase in the number of plasmoblasts and early plasma cells in mitosis; grouped plasma cells; nuclear abnormalities in plasma cells consisting of multiple nuclei, nuclear fragments, and indented and lobulated nuclei; cytoplas-

mic abnormalities consisting of displacement or absence of unstained areas, marked variation in size and shape with projections and ragged streamers; relative and absolute decrease in myeloid, erythroid and megakaryocytic elements, and a tendency toward immaturity. This supports the view that plasma cells arise from reticulum, that they constitute a distinct strain of cells, and that there is no sharp line of demarcation between solitary plasmacytoma, plasma cell myeloma, and plasma cell leukemia.—*John T. Myers.*

**ELLIOTT, K. A. C.** Swelling of brain slices and the permeability of brain cells to glucose. *Proc. Soc. Exp. Biol. & Med.*, 63, 234-6. 1946.

Slices of rat brain cortex swell in isotonic saline more than do similar slices of liver. Brain slices swell in an electrolyte-free immersion fluid of  $2\times$  isotonic glucose, fructose or sucrose (25-58%). Such swelling is prevented by the addition of electrolytes,  $0.2-0.4\times$  isotonic, to  $1.6\times$  isotonic sugar solutions. Author concludes: "Brain tissue behaves as if freely permeable to glucose, fructose and sucrose in the absence of electrolyte but, in the presence of small amounts of salt, these sugars behave as impermeable solutes." Swelling was minimal in Ringer's solution plus 3% gelatin, in blood serum, in isotonic  $\text{CaCl}_2$  and in isotonic  $\text{MgCl}_2$ .—*H. A. Davenport.*

**GOMORI, GEORGE.** Staining of chromaffin tissue. *Amer. J. Clin. Path., Tech. Sect.*, 10, 115. 1946.

Heidenhain's azocarmine modification of Mallory's aniline blue collagen stain is suggested as a satisfactory specific stain for chromaffin tissue. The following procedure is recommended for paraffin sections fixed in aqueous non-dichromate fixative solution, rather than in alcoholic fixatives:

Remove paraffin in xylene and pass through ethanol to water; stain 60-90 min. at  $56-60^\circ\text{C}$ . in 0.05% solution of azocarmine G in 1% acetic acid; rinse in tap water, blot and rinse in 95% ethanol; differentiate under microscopic control in 1% solution of aniline in 90-95% ethanol to point where chromaffin cells are a deep pink shade against paler cortical cells, requiring 5-60 min. depending on type of fixation and thickness of section; rinse in tap water; immerse 20 min. in 2-3% solution of phosphotungstic acid; wash 1 min. in tap water; stain 15-40 min. in Mallory's mixture of aniline blue and orange G (quinoline yellow or tartrazine may be substituted for orange G with better contrast) until connective tissue is deep blue; rinse in tap water; dehydrate, clear and mount.

Chromaffin granules of human adrenal medulla stain bright purplish red against a dull orange (or greenish yellow using quinoline yellow or tartrazine) of cortical cells. Rat and rabbit chromaffin tissue stain a more bluish purple color. In tumors chromaffin granules range in color from bright ruby red to almost clear blue.

Granules in alpha cells of pancreatic islets, and in some cells of anterior pituitary, granulations of human neutrophil leucocytes and of fresh myelocytes and granules of entero-chromaffin cells of guinea pig and man stain similarly to those of chromaffin tissue of adrenal medulla.—*L. Farber.*

**HIGHMAN, B.** Improved methods for demonstrating amyloid in paraffin sections. *Arch. Path.*, 41, 559. 1946.

Amyloid in paraffin sections fixed in formaldehyde may be demonstrated satisfactorily by either of the following methods:

(1) Deparaffin in xylene, and pass through graded alcohols to water; stain 5 min. by Weigert's iron-hematoxylin-acid method; wash in water; stain 1-5 min. in 0.5% crystal violet or methyl violet in 2.5% acetic acid; wash in water and mount in modified Apathy's syrup prepared by dissolving 50 g. each of gum acacia and cane sugar and 10 g. of  $\text{NaCl}$  in 100 ml. of water with 1.5 ml. of 1% merthiolate as preservative. Amyloid, cartilage and some types of mucoid material stain purplish to violet red against a bluish background. Nuclei are sharply stained by the hematoxylin.

(2) Deparaffin; stain 1-5 min. (until deep red) in 0.5% Congo red or Congo corinth G (Colour Index No. 375) in 50% ethanol; wash in water; differentiate 1-3 min. in 80% ethanol containing 0.2%  $\text{KOH}$ ; rinse in water; counterstain with iron or alum hematoxylin; wash in water; dehydrate, clear and mount by successive use of acetone, acetone-xylene, xylene and clarite.—*L. Farber.*

LERNER, EDWIN M. A rapid Gram stain for tissue. *Arch. Path.*, 41, 674. 1946.

Tissue, fixed 24 hours in Zenker's Solution and embedded in paraffin, is sectioned at 6  $\mu$ , mounted on slides, and heated at 56°C. for 2-6 hours. The tissue sections are then stained as follows:

Treat 10 min. with xylene, 10 min. with absolute ethanol and rinse in 95% ethanol; stain 1 min. in Sterling's gentian violet solution (5 g. crystal violet, 10 ml. absolute ethanol, 2 ml. aniline, 88 ml. distilled water); wash in water; treat 1 min. with iodine solution (1% iodine in 2% aqueous KI); wash in water, decolorize 10-30 sec. with 1:1 mixture of acetone and absolute ethanol; wash in water; counterstain 30 sec. with 1% aqueous safranin solution; wash in water; rinse in 95% ethanol to remove excess safranin; rinse in absolute ethanol and xylene; mount in balsam.

Gram-positive organisms are stained deep blue to violet; Gram negative organisms, red; nuclei, pink to red, with good detail; cytoplasm, light yellowish pink. Bacterial spores appear as clear spaces within cells.—*L. Farber.*

PROESCHER, F., ZAPATA, E. M., and MC NAUGHT, J. B. A rapid method of staining tissue sections for immediate diagnosis. *Amer. J. Clin. Path., Tech. Sect.*, 10, 50. 1946.

The staining method is similar to the hematoxylin-eosin technic in results, but differs mainly in specifying azophloxine (protoplasmic stain), celestin blue (nuclear stain) and euparal (for clearing and mounting).

Staining solutions used are as follows: 1) 0.1% aqueous azophloxine (CI No. 31); 2) an aqueous solution containing 1.0% celestin blue (CI No. 900), 10.0% ferric ammonium sulfate, 14% by vol. glycerol and 2% by vol.  $H_2SO_4$ . This is prepared by the following procedure: add the celestin blue and ferric alum to the water; boil 5 min. with constant stirring; cool, filter and add the glycerol and  $H_2SO_4$ ; make up to volume. Euparal is a mixture of comsol, eucalyptol, paraldehyde and sandarac with a refractive index of 1.43 and is obtainable from Arthur H. Thomas Company of Philadelphia.

The following procedures are recommended:

**Fixation of tissue**—place 5 mm. thick piece of tissue in a 50 ml. Pyrex test-tube containing 15-20 ml. of 10% formalin solution and boil over an open flame for 30 sec.; pour into small basin of water, from which wet tissue is placed on block holder of microtome and partially frozen.

**Sectioning**—section tissue, just before it is frozen hard to avoid brittleness, 15  $\mu$  thick, remove from knife with wet finger, place in small basin of tap water, dip up a flat intact section in a small wire-gauze basket for sectioning. Wire basket is made by forming a circular 2.5 cm. loop of stiff copper wire, leaving a 3 cm. handle at right angle to the loop and soldering the free end to complete the circle; a fine bronze wire gauze is soldered about the bottom of the circle forming a basket.

**Staining**—prepare stain just before use by mixing solutions 1 and 2 in proportion of 2:1 and pour into a Syracuse type of watchglass. Place the basket with wet section in the stain for 10-30 sec., then in basin of tap water so that stained section is freed from basket, pick up section on glass slide and blot free of water with dry cloth. The addition of 1-2 drops of 95% ethyl alcohol on stained section in basket before dipping into water makes it flatten out more easily.

**Dehydration**—Flood slide with 95% ethyl alcohol and drain, flood with absolute alcohol and drain, place drop of euparal on middle of section, cover with cover slip and examine.

Sections are permanent and comparable with results obtained by paraffin procedure using hematoxylin and eosin, but with less marked shrinkage.—*L. Farber.*

ROMANES, J. G. A new silver method for staining paraffin sections of the nervous system. *J. Anat.*, 80, 205-7. 1946.

At Cambridge only Bayer's pre-war protargol has proved satisfactory for Bodian's (1936) method for staining paraffin sections of nervous tissue. Because of the difficulty in obtaining this substance, another method, using colloidal silver solutions, was sought.

The method devised is as follows: Fix nervous tissue in neutral formalin. Pass sections through benzene; embed in paraffin. Attach to slides with albumin. It is recommended that the sections be floated on alcohol (70%), blotted with cigarette paper, and dried on a hot plate. Bring sections to water, tap or distilled, then place in the impregnating fluid for 4-24 hr. at 58° C. Rinse rapidly in water. Develop for 5 min. in: Pyrogallol, 1 g.; hydroquinone, 1 g.; sodium sulphite cryst., 10 g.; distilled water 100 ml. Wash well in water. Stages 1-4 may be repeated if more intense impregnation is required. Then transfer to 0.3% gold chloride in 2% glacial acetic acid for 5 min., rinse in distilled water, and leave 10-15 min. in 2% oxalic acid in 1% formalin. Wash well, treat with 5% hypo, 5 min., dehydrate, clear and cover.

The impregnating solution is prepared as follows: Dissolve 1 g. of AgNO<sub>3</sub> in 20 ml. of distilled water and just enough dilute ammonia to dissolve the ppt formed by the first few drops. Dilute to 1 l. with distilled water. This forms a stock solution. To 30 ml. of above solution add 10 ml. of 1% gelatin in fluid condition (slightly warmed), 0.8 ml. of 0.5% tannic acid in distilled water and 0.3 ml. of pure pyridine, stirring the solution all the time. This solution, which gradually grows dark brown due to formation of colloidal silver, should be made up fresh shortly before use and discarded after impregnation.

After correct impregnation the sections show purplish-black fibers on a clear background and reddish cells with well-marked neurofibrils. Boutons are not well shown. With cerebral cortex, 10% chloral hydrate should be added to the 4% formaldehyde used for fixation.—*Warren Andrew.*

**STENGER, ALBERT H., and CHARIPPER, HARRY A.** A study of adrenal cortical tissue in *Rana pipiens* with special reference to metamorphosis. *J. Morph.*, 78, 27-42. 1946.

For studies on the adrenal cortex of the frog during several stages of metamorphosis, Bouin's, Flemming's (the stronger fluid without acetic acid), Champy's and Helly's fluids were used as fixatives. The last two solutions were of special value in identifying adrenal cortical tissue, because of the blackening action of the osmium on the lipid granules of the cortical cells. Post-chromation in 3% aqueous potassium bichromate, or use of the Champy-Kull technic, facilitated identification of chromatin cells.

Dawson and Friedgood's azocarmine technic after Champy or Flemming fixation and post-chromation proved most satisfactory. Early cortical cells were stained red, late ones blue; Stilling cells a brilliant carmine; cells of the medullary adrenal orange or brown.—*Elbert C Cole.*

**ZORZOLI, ANITA.** Effects of vital dyes on early development of the amphibian embryo. *Proc. Soc. Exp. Biol. & Med.*, 63, 565-9. 1946.

Nile blue sulfate, neutral red and Bismark brown in concentrations of 1:10,000 to 1:1,000,000 were studied for their effects on developing eggs of *Rana pipiens*. Critical concentrations, below which normal development occurred were: N. B. S., 1:750,000; N. R., 1:250,000; B. B., 1:100,000. The gastrulation stage was most vulnerable, with stoppage of growth or lag of the animal hemisphere with the result that masses of yolk material remained exposed. Eggs in the 2-cell stage were exposed for timed intervals (3-67 hr.) in graded dye concentration and then were transferred to fresh spring water. Normal development followed by abnormal was seen at gastrulation. Exposure of embryos at various stages of development showed maximum effects at stages 9 and 10, decreasing at 11 and 12, and no abnormalities produced at stage 14. Dye solutions buffered to pH 4.5 were without effect while those of pH 6.3 to 8.5 gave the same results as unbuffered dyes.—*H. A. Davenport.*

## PLANT MICROTECHNIC

**JONES, RICHARD C.** The Feulgen reaction as a cytological technique with *Allomyces arbuscula*. *Mycologia*, 39, 109-12. 1947.

Young plants of *Allomyces arbuscula* were fixed in each of four fixatives for varying lengths of time. The fixatives used were: (1) Feulgen's, (2) Carnoy's A, (3) Carnoy's B, and (4) a Meve's modification of Flemming's solution. Formulae

and a summary of staining technic are included in the article. The following procedure was found to give satisfactory results: fixation in Feulgen's or Carnoy's B; hydrolysis; 5-20 hr. staining in fresh basic fuchsin solution; bleaching in three dips of Johansen's aqueous solution of  $\text{SO}_2$ ; dehydration in an alcohol series; clearing in xylene; mounting in Canada balsam.—*Elbert C. Cole.*

### MICROÖRGANISMS

**DIERCKS, F. H., and TIBBS, R. O.** A rapid method for the staining of *Rickettsia orientalis*. *J. Bact.*, **53**, 479, 480. 1947.

McNeal's tetrachrome stain, prepared according to the directions of the manufacturer, has been used for routine staining of air-dried smears of yolk-sac material infected with *Rickettsia*. The method is as follows: Fix and kill in absolute methyl alcohol for 3-5 min.; prepare dye solution by adding 4-5 ml. of stock solution to 75 ml. distilled water; transfer slides directly from fixative to stain with gentle agitation and leave for 15-20 min.; clear by dipping in absolute acetone until stain no longer dissolves; blot dry, examine under oil-immersion lens using a blue ground-glass filter. The background is pinkish; *Rickettsia* are dull blue; bacterial contaminants are bright blue; and leucocytes and erythrocytes are differentially stained. This method is not as good as the one employing Giemsa stain for photographic work but is faster and gives more consistent results when used by ordinary laboratory helpers. It is not recommended for use on animal tissue smears.—*V. Kavanagh.*

**DYAR, M. T.** A cell wall stain employing a cationic surface-active agent as a mordant. *J. Bact.*, **53**, 498. 1947.

The cell wall of bacteria and yeasts was stained as follows: Heat-fix a smear; add 3 drops M/100 (0.34%) cetyl pyridinium chloride; add 1 drop saturated aqueous Congo red, mixing on slide; wash; counterstain with methylene blue (no specifications given); wash; examine in water under a cover slip or in oil. The cell wall is red, the cytoplasm is blue.—*V. Kavanagh.*

**HENDERSON-BEGG, A., and FULTON, FORREST.** The standardization of a scrub typhus vaccine. *J. Path. & Bact.*, **58**, 381-9. 1946.

For a quick routine staining of rickettsiae the following procedure may be used: Fix light impression smears of the cut surface of lung with gentle heat. Treat with *N* HCl, 2 min. (this enhances stainability with basic dyes); wash and stain 30-60 min., with 1:2000 polychrome methylene blue (polychromed by heating 45-60 min. at 56°C. with 1%  $\text{Na}_2\text{CO}_3$ ; on cooling neutralized with *N* HCl and filtered; diluted 1:20 before use).

To stain rickettsiae a different color from cells, prepare smears of uniform thickness and free from blood. Fix with gentle heat and treat for 10-15 sec. with 10% HCl. Wash and stain 2 min. with boiling carbol fuchsin (Ziehl-Neelsen diluted 1:5); and wash. Differentiate and counterstain with 1% malachite green, 30-60 sec. (This stage is critical.) *Rickettsiae* stain deep reddish purple against a greenish blue background of cytoplasm and nucleus. The same method is suitable for material fixed in formol-Muller; after staining with boiling carbol-fuchsin, the sections are decolorized 10-15 sec. with 5% citric acid and counterstained 10-25 sec. with malachite green.

To determine the rickettsial content of vaccine, stainable rickettsiae are counted by the following technic. Mix a small amount of vaccine with an equal volume of a formalin-killed suspension of *Clostridium welchii* of accurately determined concentration (homogenized by spraying through an atomizer). Dry the mixture on a slide; fix the dry film with heat. Treat 5-10 min. with 40% formalin, wash and stain 2 min. with boiling carbol-fuchsin. Wash; decolorize 10-15 sec. with 5% citric acid. Counterstain 10-25 sec. with 1% malachite green. *Rickettsiae* stain red, *Cl. welchii* deep crimson and the background pale greenish blue. The rickettsiae are generally clumped, hence for the most accurate counts the clumps should be broken up by spraying; this method is too tedious for routine use.—*S. H. Hutner.*

JOANNIDES, GEORGE. A simple method of staining malarial parasites. *J. Lab. & Clin. Med.*, 32, 89. 1947.

The following method is satisfactory for staining malarial parasites: Place 0.5 g. methylene blue and 1.5 g. borax in a 50 ml. porcelain dish. Add 30 ml. of boiling tap water with stirring, place over a small flame and evaporate almost to dryness at 75–80° C. Repeat this twice. Let stand at room temperature 24 hr.; without stirring add 30 ml. of 95% alcohol. After 5 minutes pour the alcohol into a 100 ml. bottle and let the residue dry almost completely. Repeat the extraction once with 30 and once with 40 ml. of alcohol. Place 15 drops of this alcoholic stain on an unfixed blood film. After 30–60 sec., add 15 drops of a 1–2000 solution of eosin in tap water, and mix well with a glass rod. After 3–4 min. flood with tap water, wash, and let dry. Red cells are light green, slightly pinkish; eosinophilic granules are pink; the remaining cells and malarial parasites stain as with Giemsa stain.—*John T. Myers.*

LILLIE, R. D. Reactions of various parasitic organisms in tissues to the Bauer, Feulgen, Gram, and Gram-Weigert methods. *J. Lab. & Clin. Med.*, 32, 76–88. 1947.

Sharply stained nuclei were demonstrated by the Feulgen method in the following: *Sarcosporidia*, *Toroplasma*, *Klossiella muris*, *Trypanosoma cruzi*, *Plasmodium gallinaceum*, in eggs, larva and adults of *Schistosoma japonicum* and *S. mansoni*; in larvae and adults of tapeworms, round worms, and *Onchocerca volvulus*, and in eggs and larvae of *Capillaria hepatica*. Poorly stained or dubiously Feulgen positive nuclei were encountered in Encephalitozoon, *Eimeria stiedae*, *Endameba histolytica*, *Torula histolytica*, *Blastomyces*, and *Histoplasma capsulatum*. The mycelial fungi, bacteria, Rickettsiae, *Coccidioides immitis*, and *Haplosporangium parvum* failed to present Feulgen staining material. The Weigert-anilin-Gram technic gave more Gram positive organisms than did the Gram-acetone method. Bauer-positive polysaccharides digestible with ptyalin and with malt extract form the capsules of *Endameba histolytica*, *Eimeria stiedae*, most *Sarcosporidia*, and are present in the egg cytoplasm of *Capillaria hepatica*. Bauer-positive material is also present in *Monilia candida*, *Histoplasma capsulatum*, *Torula histolytica*, *Haplosporangium parvum*, *Coccidioides immitis*, and *Blastomyces*. *Mycetoma*, certain coarse mycelial fungi as *Penicillium* and *Aspergillus*, the shell of the eggs of *Schistosoma*, and the opercular plugs of *Capillaria hepatica*, also in some *Sarcosporidia*, and a few individuals of *Klossiella muris*, and in *Toxoplasma*. The Bauer method is valuable in detecting scarce amebae and yeasts in tissue.—*John T. Myers.*

PIJPER, A. Shape and motility of bacteria. *J. Path. & Bact.*, 58, 325–42. 1946.

This paper propounds the revolutionary thesis that propulsion in bacteria is by spiral movements of the cell and that flagella are somewhat accidental passive appendages. Most of these observations were carried out by means of dark-field microscopy using sunlight as light source.

Bacteria may be slowed for observation by means of methylcellulose ("methocel", Dow Chemical Co.). The method is as follows: Prepare graded solutions (1.5 to 2%) of methylcellulose and add a drop to a slide using a 5 mm. loop. Gently rub on a drop from a 1 mm. platinum loop taken from a very motile broth culture. Add a cover slip and seal. The 4000 centipoise type of methocel appeared the most useful. Unlike gelatin and gums, methocel does not precipitate on bacteria or produce artifacts.

The author believes that these findings account for most failures to stain flagella in motile cultures.—*S. H. Hutner.*

## HISTOCHEMISTRY

DUNN, R. C. A hemoglobin stain for histologic use based on the cyanol-hemoglobin reaction. *Arch. Path.*, 41, 676. 1946.

The cyanol-peroxidase reaction is specific for hemoglobin, erythrocytes and hemoglobin-containing renal casts and globules. Paraffin sections of tissues fixed in 4% formaldehyde solution buffered to pH 7.0 as well as frozen sections may be used.

The cyanol stock solution is prepared by dissolving 1 g. of cyanol (C.I. No. 715, National Aniline) in 100 ml. of distilled water, adding 10 g. of zinc powder and 2 ml. of C. P. glacial acetic acid. The mixture is boiled until complete decolorization occurs. This solution is stable for several weeks. The working reagent is prepared just before use by filtering 10 ml. of stock solution, adding 2 ml. of glacial acetic acid and 1 ml. of 3%  $H_2O_2$ .

Sections are stained as follows: deparaffin and hydrate sections; stain 3-5 min. in freshly prepared working reagent, rinse in water; counterstain 1 minute in 0.1% safranin in 1% acetic acid; rinse in water; dehydrate with alcohols; clear in xylene and mount in clarite.

Hemoglobin is stained dark blue to bluish gray, the nuclei red and the cytoplasm light pink.—*L. Farber.*

**McMANUS, J. F. A.** **Histological demonstration of mucin after periodic acid.** *Nature*, 158, 202. 1946.

This note describes the histological demonstration of mucin by Schiff's reagent following the action of periodic acid. Zenker-formol sections were passed to water, after iodine and hypo, and placed for 2 min. in a 0.5% solution of periodic acid in distilled water. The sections were then washed in tap and distilled water and placed in Schiff's reagent for 15 min. at room temperature. The customary rinsings in sulphurous acid, as for the Feulgen test, followed, and the sections were dehydrated in alcohols and mounted in balsam after xylene.

The mucus of the goblet cells of the human intestine and bronchus colored strongly, as did mucous salivary glands, certain pituitary cells, the colloid of the pituitary stalk and thyroid, granules in some nerve cells in the medulla of the rat and in the human intestine, and the basement membranes of the tubular epithelium and of the glomerulus in the kidney.—*J. F. A. McManus.*

**SEMMENS, C. S.** **Cytology and microchemistry.** *Microscope* 6, 58-61. 1946.

"Microchemistry" as here used implies the biochemical study of such microscopically demonstrable structures as membranes, chondriosomes, chromosomes and nucleoli. Extracts from such physically dissimilar sources must differ, even if only slightly, in their ultimate chemical constitution. While the direct study of living material will be the ideal ultimate procedure, the many technical difficulties still to be surmounted make fixed material more promising of immediate results. Because of their relative simplicity, Levitsky's fixative and alcohol-acetic mixtures are suggested for preparing material for biochemical study.—*C. E. Allen.*



# STAIN TECHNOLOGY

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## IMPROVEMENTS IN THE PARAFFIN METHOD

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**ABSTRACT.**—Dilute hydrofluoric acid alone and in conjunction with glycerin and ethyl alcohol was employed successfully to soften various types of refractory plant materials embedded in paraffin. Serial sections were cut at 6–8  $\mu$  and no appreciable deleterious effects on cell walls, cell contents, or staining procedures occurred. “Tannins” and “phlobaphene compounds” can be removed from tissues softened by this method by treating the sections for 12–48 hours with an aqueous solution of chromic acid, potassium bichromate and glacial acetic acid prepared according to the formula given by Johansen (1940).

### I. SOFTENING OF REFRACTORY PLANT MATERIAL EMBEDDED IN PARAFFIN

Certain plant materials, as well as animal tissues, tend to be extremely difficult to section after being embedded in paraffin. Methods utilizing mixtures of glycerin-alcohol and glycerin-anilin-oil, have already been recommended for softening embedded animal tissues (Baker, 1941; Lendrum, 1944). In a modified form these methods can also be adopted for plant tissues. Exposing embedded plant material to warm water, as suggested by Johansen (1940), Sass (1940), and Ball (1941), has also been advocated but this technic may result in the serious maceration of tissues, especially if the treatment is prolonged.

It has been assumed rather generally that difficulty in sectioning such material as woody stems, coriaceous leaves, roots, etc., was produced by the lignin and silica in the walls of fibers, sclereids, and tracheary elements. Kerr (1934), however, has shown that failure to microtome woody materials successfully is due largely to the condition of the cellulose in the walls rather than to their mineral and lignin content. According to Kerr, the softening action of hydro-

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fluoric acid on woody tissues "is associated with a partial degradation of cellulose and the formation of hydrocellulose". This results in a change "in the physical properties of the fibers".

In the light of the above methods, the writers have tested the softening action of hydrofluoric acid, either alone or in conjunction with glycerin-alcohol, on a wide range of plant tissues after embedding in paraffin. Specimen vials lined with paraffin were used and prior to treatment a small area of the embedded tissue was exposed by trimming with a razor blade. All materials used in these experiments were embedded in "Tissuemat". The composition of the various solutions and the exposure times are presented in the following table.

TABLE 1. COMPOSITION OF FLUIDS AND EXPOSURE TIMES.

Schedule	Composition	Exposure-period Room Temp. 22°C.±
1.	10-15% hydrofluoric acid	3 to 7 days
2.	20 ml. glycerin 80 ml. of 70% ethyl alcohol followed by: 10% hydrofluoric acid	1 week 3 to 7 days
3.	10 ml. glycerin 10 ml. hydrofluoric acid 80 ml. 95% ethyl alcohol	3 days to 2 weeks

The exposure times listed in the above table proved satisfactory for the authors' materials but doubtless will vary depending upon the "hardness" of the tissues in each case. The softening action of the reagents may be accelerated by placing the specimen vials in a 32° C. electric oven. Tests with a sharp razor blade should be made at intervals until the desired texture is obtained.

Any of the above schedules appreciably softened the materials investigated but schedules 2 and 3 usually yielded smoother ribbons, apparently because of the "lubricating" action of the glycerin. Material allowed to remain in aqueous hydrofluoric acid for long periods may show signs of maceration. This may be alleviated by the use of ethyl alcohol. Schedule 3 is recommended for general use. It has been employed successfully in microtoming such material as the coriaceous leaves of *Mouriria Huberi* Cogn. (Foster, 1947) and the scaly winter buds of species of *Drimys*, *Pseudowintera*, *Illicium* and *Trochodendron*.

This procedure has been followed by successful staining. The writers have followed the tannic acid, iron chloride, safranin schedule of Foster (1934). In addition, the common fast-green-safranin

schedule is not altered. No visible alterations or deleterious effects of these treatments have been observed in the plant tissues so treated.

## II. THE REMOVAL OF TANNINS AND PHLOBAPHENE COMPOUNDS FROM PARAFFIN SECTIONS

Often associated with processed material which is difficult to section is the presence of tannins and phlobaphene compounds. This is particularly true of many tissues in bud scales, leaves, stems and roots. Johansen (1940) has described a method, originated by Dr. Palmer Stockwell, of treating paraffin sections prior to staining. The formula is as follows:

Chromic acid . . . . .	1 g.
Potassium biochromate . . . . .	1 g.
Glacial acetic acid . . . . .	10 ml.
Distilled H <sub>2</sub> O . . . . .	90 ml.

Depending on the material in question, it has been found that 12 to 48 hours is required to remove adequately the tannin compounds. Slides should then be washed in running water for 1 to 2 hours before proceeding to the usual staining schedules.

This procedure is of great value in histogenetic studies since there is a tendency for the embryonic layers of certain leaves, bud scales, and young stems to accumulate "tannins" to the point of obscuring nuclear divisions and plastid structure. Following this treatment, staining is noticeably improved. If the maximum time of treatment is required, there is a tendency for the sections to drop off unless the adhesive described by Sass (1940 p. 50-51, Formula III) is used.

The potency of the bleaching fluid decreases with age so that a fresh solution should be prepared after marked oxidation has occurred.

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## THE STABILITY OF SO-CALLED AXONAL ACID PHOSPHATASE AS DETERMINED BY EXPERIMENTS IN ITS "STAINABILITY"

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**ABSTRACT.**—Since the histochemical method for exhibiting acid phosphatase in bodily tissues is said to depend upon the enzyme acting on suitable substrates, it is possible to test its stability by various tests. It has been found that the background element or elements, whatever they may be, concerned with the "staining" properties of the reaction are very stable and somewhat resistant to destruction. So-called acid phosphatase in spinal cord axons has not been inactivated by subjecting it to various fixing solutions, changes in temperature and pH, relatively prolonged post-mortem autolysis nor by well known enzyme inactivators. It is believed that the "staining" reaction may be dependent fundamentally on other factors than enzymatic activity.

Since Gomori (1941) introduced his histochemical method for detecting acid phosphatase in bodily tissues, an increasing number of investigators have utilized this approach on normal or pathological tissues of different animals. A number of speculations have been made concerning its possible functional and pathological significance. The method is said to depend on the action of the enzyme, when present in tissues, to split the phosphate radical from a suitable substrate (sodium glycerophosphate and others) contained in a solution having an acid buffer mechanism and lead nitrate. The hydrolyzed phosphate ion is supposed to combine with lead to form lead phosphate at the exact site of enzymatic activity. Subsequent treatment with ammonium sulfide converts any lead phosphate formed to lead sulfide which has a distinct brown coloration. Acid phosphatase, like other enzymes, is thought to be very sensitive to temperature and pH changes and certain other inactivating substances. In regenerative studies of the sciatic nerve in the cat, it was noticed that this so-called enzyme "stained" following injection of 40% formalin into the nerve with subsequent immersion in acetone; as a result, its stability was tested further by other experiments.

<sup>1</sup>With the assistance of Miss Margaret Powers, technician.

## METHODS

A list of the methods used to test the durability of so-called acid phosphatase in axons of the brain stem and spinal cord of cat, monkey and man is given below:

## 1. The effect of fixatives:

- (a) 10% formalin—fresh tissues fixed for three days.
- (b) 40% formalin (commercial)—fixation time, one day.
- (c) Protargol fixative—butyl alcohol, 65 ml.; propyl alcohol, 25 ml.; formic acid, 5 ml.; and trichloroacetic acid, 5 ml. Fixation time, three days.
- (d) Ranson's pyridine silver fixative—absolute alcohol, 98 ml.; and ammonium hydroxide, 2 ml. Fixation time, two days.

## 2. The effect of immersion of acetone-fixed sections in fluids with extreme pH ranges.

- (a) 1% hydrochloric acid—one hour.
- (b) 1% nitric acid—one hour.
- (c) 1% ammonium hydroxide—one hour.

## 3. The effect of temperature.

- (a) Heating fresh tissue in warmed acetone (39° C.)—for ninety-six hours.
- (b) Heating fresh cord tissue in water at 65° C. for periods up to three hours, then fixation in cold acetone.
- (c) Incubation of acetone-fixed sections at 65° C. instead of 37°—for periods varying from one to twenty hours.
- (d) Boiling acetone-fixed sections in water—for one hour.
- (e) Refrigeration of fresh tissue at 4° C.—for various intervals up to thirteen days followed by fixation in cold acetone.
- (f) Effect of room temperature on fresh unfixed tissue—up to forty-eight hours followed by fixation in cold acetone.

## 4. Effect of enzymatic inactivating substances.

- (a) Immersion of acetone-fixed sections in 10% hydrogen peroxide solution—for one hour.
- (b) Immersion of acetone-fixed sections in 10% solution of phosphotungstic acid—for one hour.
- (c) Immersion of acetone-fixed sections in 10% solution of mercuric chloride—for one hour.
- (d) Immersion of acetone-fixed sections in 1% solution of picric acid—for one hour.

The methods used for "staining" the so-called enzyme were two in number: Gomori's original incubating mixture and one which employ-

ed ascorbic acid as an additional compound. The exact procedure of each is listed below:

*Gomori's Method.*

Molar acetate buffer pH 5. . . . .	30 ml.
Aqueous lead nitrate (5%) . . . . .	10 ml.
Distilled water. . . . .	60 ml.
Alpha sodium glycerophosphate (52%) in 2% aqueous solution. . . . .	30 ml.

*Ascorbic Acid Solution.*

Molar acetate buffer, pH 4.7. . . . .	10 ml.
Aqueous lead nitrate (5%) . . . . .	4 ml.
Distilled water. . . . .	60 ml.
Alpha sodium glycerophosphate (52%) in 2% aqueous solution . . . . .	12 ml.

Just before incubation, 2 ml. of ascorbic acid (Cevalin, Lilly) is added to the above solution.

The normal incubation temperature for both of these methods is 37° C. The incubation times used varied from one to ninety-six hours.

## RESULTS

The results of the study indicate that the substance which is "stained" in the axis cylinders of the central nervous system by the two histochemical technics for exhibiting acid phosphatase is remarkably stable and somewhat resistant to destruction. None of the methods employed to test its stability have completely inactivated it.

Positive presence of the so-called enzyme has been obtained following fixation with 10 and 40% formalin (Fig. 2), Ranson's fixative, and that which is used in the protargol technic.

Immersion in fluids having extreme pH ranges, such as 1% ammonium hydroxide, 1% nitric acid, or 1% hydrochloric acid for one hour, did not destroy or inactivate the so-called enzyme (Fig. 3 and 4).

The "stainable" substance also withstood ranges in temperatures well. Positive results were obtained after heating fresh cord tissue for ninety-six hours in warm acetone at 39° C.; heating fresh, unfixed cord tissue in water at 65° C. for three hours (Fig. 6); incubating acetone-fixed sections at 65° C. and boiling acetone-fixed sections containing the so-called enzyme for one hour (Fig. 1). In an animal which was kept in a refrigerated ice box, having the constant temperature of 4° C., the "stainable" substance was partly preserved up to thirteen days. Very slight breakdown began in the axis cylinders of

the cord after twenty-four hours, especially in the largest axis cylinders, and gradually continued. The changes were more rapid in the specimen left at room temperature; the so-called enzyme was considerably broken down after twenty-four (Fig. 5); at forty-eight hours, a few axons still "stained". In all of the cases studied the axons located in the posterior funiculus seemed to be more resistant to temperature changes than in other portions of the cord.

Immersion in such well known enzymatic inactivating solutions as hydrogen peroxide, phosphotungstic acid, picric acid or mercuric chloride did not affect the "stainable" substance to any material degree. If anything, they actually seemed to enhance the intensity of the "staining".

The addition of ascorbic acid or vitamin C (Cevalin, Lilly) to the incubating mixture seemed to speed up or vitalize the reaction considerably. A positive reaction was obtained by using ascorbic acid alone without the use of sodium glycerophosphate or any other substrate. With the addition of ascorbic acid to the incubating solution, it took on the average, about one-fifth as long as Gomori's method to exhibit adequately the "stainable" substance.

#### COMMENTS

Most enzymes are easily affected by changes in temperature, hydrogen-ion concentration, heavy metals, phosphotungstic and picric acids and oxidizing agents. Some of the common solvents for enzymes are water, dilute ethyl alcohol and dilute acids and alkalis.

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#### EXPLANATION OF FIGURES

Photomicrographs taken under high power showing the presence of so-called acid phosphatase in spinal cord axons of cat following various experimental procedures. All sections incubated at 37° C.

1. Following sixty minutes boiling in water of acetone-fixed sections—21 hours incubation in ascorbic acid solution.

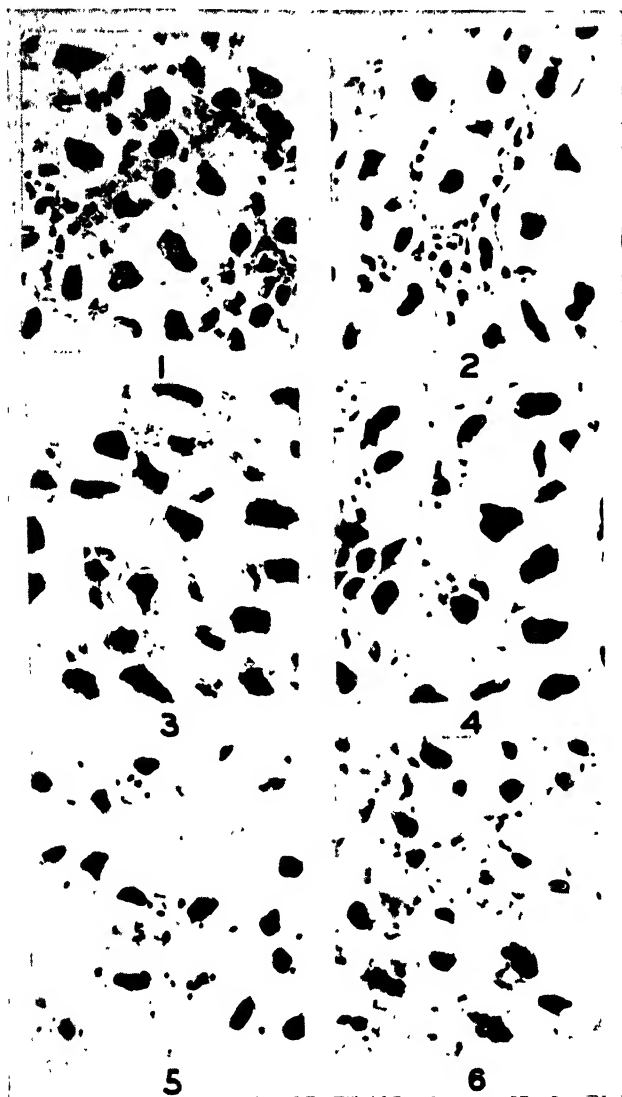
2. Following fixation in 40% (commercial) formalin—96 hours incubation in Gomori's solution.

3. Following immersion of acetone-fixed sections in 1% ammonium hydroxide for one hour—24 hours incubation in ascorbic acid solution.

4. Following immersion of acetone-fixed sections in 1% nitric acid for one hour—24 hours incubation in ascorbic acid solution.

5. Following exposure of spinal cord to room temperatures (65° F.) for twenty-four hours—acetone-fixed sections incubated for 24 hours in Gomori's solution.

6. Following three hours heating of fresh cord tissue in water at 65° C., then fixation with acetone—incubation for 5 hours in ascorbic acid solution.



Hawk, Oser and Summerson (1947) state that the majority of enzymes are inactivated by heating them in solution to about 60° C. This involves a denaturation of protein.

The "stainable" substance in the axons, whatever it is, seems to be exceedingly stable and somewhat resistant to destruction. Nearly all of the above criteria and methods have been applied to testing the stability of the so-called enzyme acid phosphatase, by the histochemical method. These have failed to inactivate or destroy the substance which is "stained" by the technic. In several trials, the reaction was also positive in the presence of ascorbic acid but in the absence of sodium glycerophosphate or any other substrate. The response of the reaction to increase in the temperature of the incubating solution suggests that it may be chemical in nature. If this is true, it may possibly be developed into something which will be a valuable addition to general staining procedures.

### CONCLUSIONS

1. The axonal substance in neurons of the brain and spinal cord, which is "stained" by the histochemical methods designed to exhibit activity of the enzyme, acid phosphatase, is exceedingly stable and somewhat resistant to destruction.

2. It withstands the action of various fixatives, extreme ranges in pH and temperatures, known inactivators and relatively prolonged post mortem autolytic activity.

3. Certain phases of the investigation on the material tested suggest that the reaction fundamentally may depend upon chemical rather than enzymatic phenomena.

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## DIFFERENTIAL STAINING OF TISSUE IN THE BLOCK WITH PICRIC ACID AND THE FEULGEN REACTION

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**ABSTRACT.**—The authors have found a modification of the Feulgen reaction to be a satisfactory stain for tissue in the block.

Pieces of fresh mammalian tissue not thicker than 5 mm. are fixed for approximately 48 hours at 25° C. in a mixture of equal parts of 5% aqueous sulfosalicylic acid and saturated aqueous picric acid. They are washed for 30 minutes in three ten-minute changes of distilled water and placed in Feulgen's staining solution diluted to one-half strength with distilled water. The staining solution is allowed to act for 24 hours (2 to 3 mm. thick blocks) up to 48 hours for 5 mm. thickness. After staining, the specimens are transferred to a mixture of sodium bisulfite, 0.5 g. and N hydrochloric acid, 5 ml. in 100 ml. of distilled water. Two changes of 15 to 30 min. each in the acid sulfite are given and these are followed by dehydration through 50%, 70% and 95% alcohol. One to two hours are allowed for each change except the last 95%, in which the stained tissue is allowed to remain overnight. The dehydration is completed in two changes of absolute alcohol with subsequent clearing in xylene and embedding in paraffin. Sections may be cut 10  $\mu$  or other thickness desired, mounted on slides, paraffin removed, and covered in the usual manner. Nuclei stain reddish violet against a lemon yellow background when the stain is typical. Orange G, 200 mg. per 100 ml. may be added to the fixing fluid if a more polychromatic effect is desired.

The Feulgen reaction is employed usually to stain sections. However, Margolena (1931) used it for whole-mounts of protozoa and small worms, and Liang (1947) has adapted it to staining nerve trunks in blocks of mammalian tissue.

The procedure to be described developed from experiments in which Liang's technic was used both in its original form and with essential modifications. In his method, fresh tissue is fixed in 1% aqueous formic acid for 1 to 3 hours, rinsed, stained with Schiff's reagent until the nerves appear, washed about an hour in a solution of

<sup>1</sup>Contribution No. 483.

sulfurous acid, and finally dehydrated. Whole-mounts of thin material can be made or the blocks can be sectioned after embedding in paraffin. We found the method to be very reliable for bringing out the topography of small peripheral nerve trunks which contained myelinated fibers. The leuco basic fuchsin stains myelin readily under the conditions listed above. We were unable, however, to secure staining of unmyelinated fibers.

Prolongation of the time of fixation in acid tends to bring in the staining of cell nuclei and therefore produces the typical "nucleal" reaction described originally by Feulgen and Rossenbeck (1924). This fact, coupled with the observation that the presence of picric acid used in fixation did not interfere with the staining of nuclei, suggested that a technic for block staining in two colors might be developed.

### *Material and Methods.*

Fresh tissues from rat, kitten, rabbit, dog and monkey were fixed for periods of time varying from 1.5 hours to 8 days. Spleen, intestine, intercostal muscle, trachea, esophagus, thyroid gland, spinal cord or brain, and sciatic nerve were used to give a wide variety of tissues and structures. The facility of penetration of both the picric acid used in the fixative and the subsequent Feulgen reaction was tested with pieces of spleen which measured about 1 cm. through their thinnest dimension.

Only a limited number of fixing fluids were tried because it is essential for best results that the hydrolytic effect of the acid used in fixation be adequate to bring out the Feulgen reaction and yet not so vigorous that inadequate time will be allowed for good penetration of the picric acid. Since the objective of the technic was to secure a bicolor or polychromatic stain in the tissue block, picric acid served both as a background stain and fixing agent. Trichloroacetic acid and sulfosalicylic acids were the logical ones to use as hydrolytic agents. Both are good protein precipitants and penetrate rapidly. They are rather highly ionized, hence strong organic acids.

The two fixing fluids used most were the following:

Sulfosalicylic acid, 5% aq.....	50 ml.
Picric acid, sat. aq.....	50 ml.

and

Trichloroacetic acid, 5% aq.....	50 ml.
Picric acid, sat. aq.....	50 ml.

In some experiments, formalin to make 5% of the volume of the acid mixture was added. When formalin was used a very thorough wash-

ing was given the tissue after fixation to remove it before placing the tissue in the staining solution. (See Bauer, 1932, on the use of formalin-fixed tissues for the Feulgen stain.) The addition of formalin seemed to improve the fixation somewhat, but its effect was not specially marked in conjunction with the rather efficient acid mixtures. Of the two acids, sulfosalicylic seems preferable to trichloroacetic since it gave more consistently good fixation and subsequent staining.

Attempts to introduce a third color into the final stain by adding either aniline blue or fast green FCF to the fixing solution were unsuccessful, since neither of these dyes penetrated the tissue for more than about 0.5 mm. However, when orange G was added to the fixative in a concentration of 0.2%, its ability to penetrate appeared to be about the same as that of picric acid.

The Schiff's reagent used for staining was not varied and was prepared in the manner recommended originally by Feulgen. Advantage was taken of the improvements suggested by de Tomasi (1936) and the method for removal of residual color with activated carbon by Coleman (1938.) The stock solution was usually diluted with an equal volume of distilled water just before use. The time of staining was varied as follows: 6, 12, 24, 48 and 72 hours. The results obtained indicated that penetration of the stain was rather slow and that there was no serious tendency toward overstaining. Hence the optimum time is not very critical but should be sufficient for complete penetration. The best results in our series appeared in the 24 and 48 hour stains. Blocks more than 5 mm. thick required at least 48 hours and spleen was frequently not fully penetrated even then. The 72-hour specimens were less well differentiated and somewhat overstained. Small blocks, such as 6 to 8 mm. lengths of rat intestine, were usually stained adequately in 24 hours.

After staining, the blocks of tissue were either treated for 0.5 to 1.0 hour in a dilute solution of sulfurous acid (formula in step 4 of method recommended below) or washed directly in water. We have gained the impression that the staining is sharper when the sulfurous acid is used between the staining bath and the final washing, which is in agreement with the recommendations given in the literature for staining sections by the Feulgen technic.

Any residues of staining solution or sulfite left in the tissue after treatment with sulfurous acid appear to be soluble in 50% alcohol, hence washing in water after this step was usually omitted, although washing in distilled water did no harm. Alkaline tap water would tend to remove the picric acid stain, likewise alkalinity would be undesirable in the alcohol used for dehydration.

The results obtained from 400 blocks of tissue processed to include the variables listed above indicate that the following technic can be recommended:

1. Fix fresh animal tissue in the following mixture for not less than 36 nor more than 60 hours at room temperature (25°C.).

Sulfosalicylic acid . . . . .	2.5 g.
Distilled water . . . . .	50 ml.

When dissolved, add

Picric acid, sat. aq. . . . .	50 ml.
Orange G (optional) . . . . .	100 mg.

2. Wash through 3 changes of distilled water, 10 min. each, to remove the excess of the fixing fluid.

3. Place in Feulgen's staining solution (Schiff's reagent) diluted with distilled water to one-half the strength of the stock solution. The stock solution is prepared as follows:

Dissolve 0.5 g. basic fuchsin by pouring over it 100 ml. boiling distilled water. Shake thoroughly and cool to 50°C. Filter and add 10 ml. 1N HCl to filtrate. Add 0.5 g.  $K_2S_2O_8$  (potassium metabisulfite) and allow to stand in the dark well stoppered for 18 hours or until it is a light straw color. To remove residual color add 0.25 g. of activated charcoal, shake for a minute or two and filter. If the solution is not clear, refilter through the charcoal. Keep the bottle well stoppered at all times.

Stain for 24 hours if blocks are small, 2-3 mm. thickness, or 48 hours for 5-7 mm. Limit thickness to 5 mm. if possible. Both fixing and staining are carried out at room temperature (about 25°C.).

4. Transfer the specimens to sulfurous acid solution:

Sodium or potassium bisulfite . . . . .	.5 g.
Normal hydrochloric acid . . . . .	5 ml.
Water . . . . .	100 ml.

Use two changes of 15-30 min. each.

5. Dehydrate through 50%, 70% and 95% alcohol, 1-2 hours each, allow to remain in a second change of 95% overnight and follow with two changes of absolute alcohol and two of xylene.

6. Embed in paraffin.

7. Cut sections 8 to 15 $\mu$  thick, mount, remove paraffin with xylene and cover in the usual manner.

## DISCUSSION

The stain shows nuclei in hues of reddish violet against a lemon yellow background when orange G has not been added to the fixing fluid. When orange G was added, red blood cells and voluntary muscle usually took it up and added thereby some degree of differen-

tiation among the nonnuclear tissue components. The shafts of hairs always stained bright greenish yellow. Epidermis and white fibrous connective tissue were pale yellow to lemon yellow. Smooth muscle was stained by the picric acid but the sarcoplasm of striped muscle stained orange. In some fibers the anisotropic bands were seen to be more darkly stained than the isotropic but the finer H and Z lines were not distinguishable. The exact hues of the polychromatic effects obtained appeared to be somewhat unpredictable, but they permitted the different tissue components to be distinguished easily.

Structures other than nuclei were stained by the Feulgen reaction. The submucosa of intestine, cartilage, the intima of blood vessels and, occasionally, the fibrils of elastic tissue stained violet. Myelin stained violet to reddish violet and always stained whether the period of fixation was 1.5 hours or 48 hours. Elastic tissue was more likely to stain after a short period of fixation but not after a long one. The nuclei of liver and striped muscle did not stain as strongly as those of tubular glands and epithelium.

As given, this method does not produce pleasing results when used with tissue of the central nervous system. Although color differentiation exists between the grey and white matter, the details of the tissues are not sufficiently defined for the usual purposes of the neurohistologist.

The ease of execution and reliability of this technic adapts it to uses which require good differentiation of nuclei in serial sections and classroom slides. Since the staining is completed in the block, the method can be substituted for hematoxylin and eosin with considerable saving of time. Like most methods, it is neither perfect nor infallible. Lack of penetration of the Schiff's reagent limits the usable thickness of the tissue to about 5 mm. Blocks of greater width and length can be used provided that the pieces are made rather thin.

The permanence of the stain has been tested only to the extent of leaving slides in direct sunlight for approximately 50 hours total exposure. The amount of fading was not noticeable when the preparations were compared with unexposed slides.

Plant material that remained in the lumen of the intestine showed typical nuclear staining.

The fact that nucleoproteins are undergoing hydrolysis during fixation, which is one of the essentials of the Feulgen reaction, makes a time schedule for the entire process necessary. This time schedule is not rigid, but gross variations from the 48-hour fixing time are undesirable. In the regular hydrolysis for the Feulgen stain with sections, the time is approximately 0.1 hour in *N* HCl at 60°C. In

the block staining process described, hydrolysis occurs at about 25° in an acidity that is slightly less than 0.1 N. If the theoretical time needed for hydrolysis in the latter process be calculated by Vant Hoff's law for temperature effects, and the law of squares for ionic activity, the following equations are used.

(1) 60°-25° = 35° difference in temperature. If the reaction time doubled for each 10°, the reaction rate at room temperature should have been  $\frac{1}{3.5 \text{ squared}}$  or approximately  $\frac{1}{12}$  that at 60°.

(2) The reciprocal of the square of the ionic concentration of H<sup>+</sup> is roughly  $\frac{1}{100}$ . Hence the theoretical rate in the block is about  $\frac{1}{1200}$  that of the customary one for hydrolyzing sections.

(3) Sections are hydrolyzed about 0.1 hour so that theory would require a period of hydrolysis in our technic with blocks of 1200 × 0.1 or 120 hours.

Experiments indicate that 120 hours is too long and that the optimum is somewhere near 48, therefore it appears that the hydrolysis does not follow strictly the above laws, or that the presence of picric acid influences the liberation of bonds in nucleoproteins and thus activates them to produce the characteristic Feulgen stain.

**Acknowledgement:** The authors wish to express their appreciation of assistance received through a research grant from the Biological Stain Commission.

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## SOME OIL SOLUBLE DYES WHICH STAIN SUBERIZED DEPOSITS IN ORANGE VESICLES<sup>1</sup>

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**ABSTRACT.**—Of 16 commercial oil-soluble dyes, not previously in common use among botanists, eight (including oil blue NA, already known as a stain for rubber) have proved better than Sudan III and Sudan IV in staining suberized deposits in orange vesicles.

Supplementary to a chemical analysis of the “fatty” constituents of citrus fruit is a study of the “fat” staining tissues of the fruit. In the course of this study it was found that the fruit vesicles had peripherally located deposits of suberin which were effectively stained by either Sudan III or IV as shown in Fig. 1. Other tissue substances

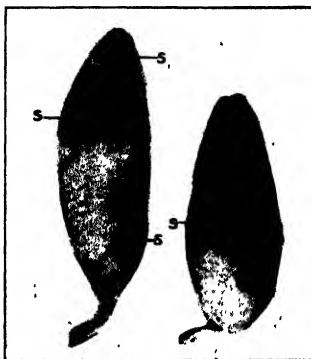


FIG. 1.—Suberized deposits, S, of orange vesicles,  $\times 5$ .

listed as having affinity for these dyes include fats or oils contained singly in tissues or as components of complex tissue impregnations as is the case for suberin and also cutin and resin (Johansen, 1940). The same dyes are also employed to stain rubber in plant tissues (Haasis, 1944).

Recently, however, a commercially used oil soluble dye, oil blue NA, was found effective in staining rubber deposits in guayule (Addicott, 1944; Whittenberger, 1944) as well as the other tissue substances

<sup>1</sup>Agricultural Chemical Research Division Contribution No. 194.

listed above as having affinity for the Sudan dyes. Subsequently, this same dye, oil blue NA was found applicable as a fat stain in animal tissues (Lillie, 1945). In the case of the suberized areas of citrus vesicles, oil blue NA gave a stain which was exceedingly more brilliant than that achieved with either Sudan III or IV. Consequently, numerous other commercially recommended oil soluble dyes were tried as suberin stains using the same fruit material, with the possibility of their having further use comparable to that of Sudan III and IV.

TABLE 1. OIL SOLUBLE DYES FOR USE AS FAT STAINS.

Common Name	Federal Name	CI No. or Identity
Oil red OS (CCC)	D&C red No. 18	No CI No. 1-xylylazoxylazo-2-naphthol
Oil red OB (AACC)		Technical dye Sample R-6033
Oil red XO (CCC)	FD&C red No. 32	No. CI No. 1-xylylazo-2-naphthol
Calco oil red (CCC)		Sample No. 1700
Rhodamine B stearate (CCC)	FD&C red No. 37	No CI No.
Alizarine irisol base (CCC)	D&C blue No. 3 and D&C violet No. 2	CI-1073
Calcogas blue NA or Oil blue NA (CCC)	Ext. D&C blue No. 5	No CI No. 1,4-bis-amylamino-anthraquinone
Spirit Blue (NACC)		CI No. 689
Oil orange SS (CCC)	FD&C orange No. 2	No CI No. 1-0-tolylazo-2-naphthol
Orange TR (CCC)	D&C orange No. 14	No CI No. 4,5,15-tribromo-2-7-dicarboxy-3,6-fluorandiol
Oil yellow AB (CCC)	FD&C yellow No. 3	CI-22
Oil yellow OB (CCC)	FD&C yellow No. 4	CI-61
Quinoline yellow—spirit soluble (CCC)	D&C yellow No. 11	CI-800
Alizarine cyanine green base (CCC)	D&C green No. 6	CI-1078

The dyes were supplied through the courtesy of the sources indicated as (CCC) = Calco Chemical Company; (AACC) = American Aniline and Chemical Company, and (NACC) = National Aniline and Chemical Division, Allied C.&D. Corp.

A staining technic found convenient was similar to that recommended for the treatment of animal tissues with fat stains (Lillie, 1944). Accordingly, the dyes were used in a 30:30:40 mixture of ethyl alcohol, acetone and distilled water, containing about 50 mg. of dye per 100 ml. of solvent. All dye solutions were filtered before using. Prior to staining, the fresh vesicles were immersed in the solvent mixture for a few seconds to prevent later precipitation of the dye in the tissue. Staining was for five minutes. After staining, the vesicles were again immersed in the solvent mixture, in 50% ethyl alcohol and finally in distilled water.

The dyes found effective in staining the suberized areas of the fruit vesicles, including oil blue NA, are listed in Table 1. Not only oil

blue NA, but also oil red XO, oil red OB, Calco oil red, alizarine irisol base, spirit blue, Calco orange SS and oil yellow AB stained the suberized areas more brilliantly and more deeply than did the Sudan dyes.

It was considered significant to record these observations for application in similar studies concerned with the demarcation of various plant substances. Frequently for such analysis, it is advantageous to have available a dye of a certain color for contrast with that of tissue areas surrounding a particular substance, and generally brilliance in staining facilitates such microscopic study.

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## PHLOXINE AS AN HISTOLOGIC STAIN, ESPECIALLY IN COMBINATION WITH HEMATOXYLIN

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**ABSTRACT.**—A staining schedule employing phloxine as a counter-stain to Erlich's acid hematoxylin is presented. Fixation is best with Zenker's fluid, although formalin can be used. The technic is similar to the standard hematoxylin-eosin formulae but because of the staining advantages of phloxine over eosin, the technic is simpler, and quicker, resulting in clearly differentiated sections which do not fade as soon as do eosin-stained slides. A brief summary of the uses of phloxine as a biological stain is given and its advantages over eosin are discussed.

### INTRODUCTION

For some time the standard staining technics for histology have included variations of hematoxylin and eosin. In a recent survey<sup>1</sup> (1945), out of 203 hospital laboratories asked, 161 used forms of hematoxylin ranging through Erlich's acid hematoxylin, Weigert's iron formula, Bohmer's, and Delafield's; 158 made use of picric, aqueous or alcoholic eosin and almost without exception used these with some modification of hematoxylin; only 8 used phloxine, combining it in most cases in the Mallory methylene blue formula (Conn, 1940, p. 155). The author has slides twenty years old in which the hematoxylin-eosin combination had been used and the histologic details are still evident. It remains true, however, that balsam has so faded the eosin that fine details are not visible and the imagination must be used in piecing together the finer cytology. A similar trouble occurs even in new sections, and occasionally slides two or three years old will begin to fade, depending upon the dye and mounting media used. It is the purpose of this paper to discuss the relative advantages of phloxine and then present a suitable technic for its routine histologic use.

Phloxine (Syn: erythrosin BB or B extra; new pink) is a member of the xanthene series and may be either a dichlor or tetrachlor tetrabrom fluorescein. It is a higher homolog of eosin and erythrosin, having none of the orange tinge noticeable in the true eosins and less fluorescence. Its Colour Index No. is 774, and it has an absorption

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<sup>1</sup>See: Autotechnicon Methodology (The Technicon Co, New York, 1945) and Whitehead (1945)

maximum of 535.7. At 28° C. it is 50.9% soluble in water and 9.02% soluble in alcohol. It is chemically allied to eosin Y by the presence of four bromine atoms (see Fig. 1).

Phloxine found early use as a bacterial stain when Conn and Holmes (1926) made use of dyes of the fluorescein group for staining dried soil films. They showed that 3', 6'-dichloro-2,4,5,7-tetrabromo fluorescein showed up as a good bacterial stain in pure culture, deeper than eosin and less acid. Later reports showed that additions

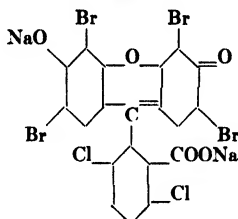


FIG. 1.—The dichlor form of a molecule of tetrabrom fluorescein.

of small amounts of calcium, aluminum, magnesium or lead salts increased the intensity of the stain (Conn and Holmes, 1928). Holmes (1929) formulated a table of solubilities of dyes. The values for phloxine and eosin in these tables are significant.

The stain since has found good use as a bacterial stain (McCalla, 1941; Maneval, 1941).

TABLE 1. RELATIVE SOLUBILITY OF PHLOXINE AND EOSIN AT 26 C°.

Phloxine C.I. 774	H <sub>2</sub> O	95% alcohol
	(Na salt) 50.90 (Mg salt) 20.84 (Ca salt) 3.57 (Ba salt) 6.01	9.02 29.10 0.45 1.17
Eosin C.I. 768	H <sub>2</sub> O	95% alcohol
	(Na salt) 44.20 (Mg salt) 1.43 (Ca salt) 0.24 (Ba salt) 0.18	2.18 0.28 0.09 0.06

Following World War I, Grüber placed a magdala red on the market which went under the names "magdalarot" and "magdalarot echt". Dr. H. J. Conn in his section on aniline dyes in McClung's "Microscopic Technique" states: "a cheaper magdala red, without qualifying designation, was also put on the market by Grüber before the war, which proves to have been (in some instances at least) a fluorescein derivative (phloxine or erythrosin) rather than a safranin." It was

such a dye that Chamberlain (1924, 1932) found stained algae better than the more expensive brand of magdala red.

Mallory, in his later work, used phloxine in his methylene blue formula. Conn (1925, p. 82) states from personal communication with Mallory that it was found to be the best *eosin* for the technic. The technic was formulated originally in 1903 and was later modified by him, in 1938 to specify phloxine instead of eosin. The formula has been used by the Biological Stain Commission as routine for phloxine samples submitted for certification.

As to variety of use, the dye is extremely flexible. Ballantyne (1940) has reported using phloxine on frozen sections with success. It is adaptable as a botanical stain, having been used successfully by

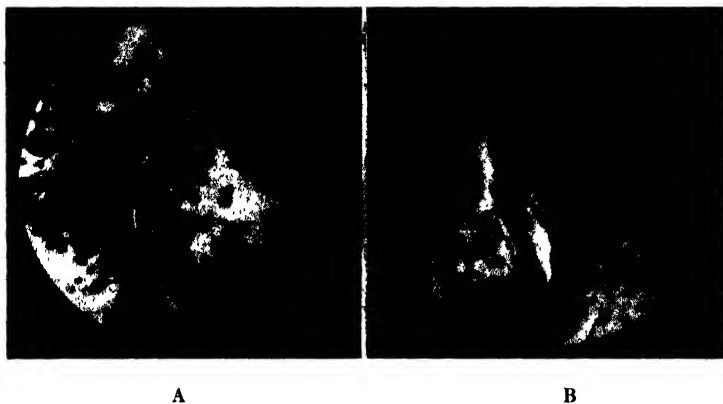


FIG. 2.—A. Testis. Human  $\times 450$ ; cut at  $15\ \mu$  and stained by the phloxine-hematoxylin technic. Note the intensity of the stain on the walls of the seminiferous tubules and developing spermatocytes.

B. Uterus. Human  $\times 450$ ; cut at about  $10\ \mu$  and stained with a standard eosin-hematoxylin schedule. Both sections were fixed in Zenker's fluid. The same light intensity was used in both A and B.

several workers. Buchholz (1938) used phloxine in combination with Delafield's or Heidenhain's hematoxylin on conifer embryos, while Stanley (1940) used the dye in studying certain of the basidiomycetes. In all, phloxine has proven itself most versatile and deserves more recognition in standard routines.

#### TECHNIC

Very little has appeared in the literature with regard to using phloxine as a counterstain to hematoxylin in tissue work. Margolina

(1938) mentions its use with Heidenhain's or Harris' hematoxylin and orange G as a differential counterstain. The following technic is similar to the standard eosin-hematoxylin technics with modifications in time and reagents:

1. Fix in Zenker's fluid.
2. Embed in paraffin and cut sections at 8 to 10 $\mu$  for best results. Mount sections on albuminized slides as usual.
3. Place in xylene for 1 minute. Place the sections in two changes of 95% alcohol for 1 minute each. Wash well in tap water. Place in iodine-alcohol for 3 minutes to remove mercuric chloride crystals. To remove the iodine color, place in a weak solution of sodium hypochlorite until the sections are bleached.
4. Place the sections in Erlich's acid hematoxylin for about 7 minutes. Overstaining will not occur, but the 7 minutes prescribed will suffice to show up most nuclei. Wash well in tap water. The solutions will stain better if a good washing is given after each of the above steps.
5. Place in Scotts Bluer ( $\text{KHCO}_3$ , 2 g.;  $\text{MgSO}_4$ , 20 g.; distilled water, 1,000 ml.) for  $\frac{1}{2}$  minute and wash well. Dip the sections in a 5% solution of acid alcohol and wash quickly in tap water.
6. Place the slides in a 5% solution (aqueous) of phloxine for 10 seconds. The dye is strong and at this strength will give a light but even distribution of pink to the cytoplasm. Wash in tap water thoroughly.
7. Place the slides in 95% alcohol for one minute and drain well. Follow the same procedure for absolute alcohol. Place the slides in a 50% solution of xylene and creosote. This has an intensifying effect upon the stain. The slide may now be placed indefinitely in xylene until the cover slip is applied. Clarite is the recommended mounting medium.
8. Results: Nuclei appear medium purple against a light pink background.

The technic is not too time-consuming, being in most cases shorter than the standard hematoxylin-eosin schedules. It is accurate; resulting in a true differentiation of the necessary histologic elements. Formalin-fixed tissue may be stained with equally good results.

The author expresses his appreciation to Dr. J. W. Denton and Mr. Fritz Carter of the pathological laboratories, New Rochelle Hospital, New Rochelle, N. Y., for introducing him to the value of phloxine as a dye in the above technic.

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## CHLORAZOL PAPER BROWN B AS A STAIN FOR PLANT TISSUES

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Herts, England*

The staining properties of Chlorazol paper brown B were discovered while a considerable range of Chlorazol dyes from Imperial Chemical Industries Ltd. (Hexagon House, Blackley, Manchester, 9, England) were being investigated with a view to their possible use as selective stains for paper fibers. The dye is not yet available from dealers.

The stain is used in saturated aqueous solution, the solubility being about 30 g. per liter at 14° C. Material may be stained overnight but in many cases, for example where the delicate cell contents are not germane to the investigation, boiling in the aqueous stain for one or two minutes will produce an equivalent depth of staining. The material is then differentiated in 1% nitric acid solution. Any solution of low pH will effect differentiation but nitric acid appears to be the best. Dehydration in acetone and mounting in Euparal have proved to be the most suitable for preserving the colors.

Trials with the stain have not been extensive but the few examples mentioned below will serve to indicate its possibilities. The first material investigated was *Clematis vitalba* L., in transverse section. By employing the boiling technic finished slides could be obtained in about five minutes. The tissues and their respective colors obtained are tabulated below:

Epidermis	Yellow
Cortex	Yellow
Pericycle (lignified tissues)	Blood-Red
Xylem	Salmon to blood-red.
Primary Phloem	Orange
Cambium	Pale yellow
Secondary Phloem	Crimson
Seive Plates	Some very bright crimson, others orange
Pith	Amber

Year old slides of sections of *Clematis* have retained their brilliancy of coloring and no noticeable fading has taken place. Sections of monocotyledenous tissues are not successfully stained by the dye.

Transverse sections of the leaf of *Ananassa sativa* stained in the dye show the epidermis red but the bundles and fibers are scarcely differentiated. Textile fibers are well stained; fine structure is brought out well. Jute, for example, stains very well and the resulting color is admirably suited for photomicrographic purposes.

F. D. Armitage has successfully stained the alga *Spirogyra* with the dye (overnight). He finds, however, that it is of no value for staining fungi, nor does it appear to be of any value for staining animal tissues.

The author wishes to thank the Council of the Printing and Allied Trades Research Association for their permission to publish this note.

## A SIMPLE METHOD FOR SQUASHING AND MOUNTING PREPARATIONS AFTER ANY STAIN

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**ABSTRACT.**—A method is described for the squashing and permanent mounting of preparations, similar to the nucleal squash technic, to be used after hematoxylin, coal tar dyes and other stains. The colored pieces are immersed in glycerin, squashed or smeared, the glycerin is slowly removed with water by capillarity and the water is afterwards similarly substituted by alcohol. The cover glass is then removed and after a rapid rinsing in alcohol the materials are permanently mounted in alcohol-soluble resin.

### INTRODUCTION

The smearing or squashing method (Nuklealquetschmethode) after a Feulgen reaction (in which the tissues are placed in a few drops of 45% acetic acid and pressed between slide and cover glass until separation of the cells and spreading of the chromosomes is obtained) is largely used in chromosome studies with good results. After smearing, the preparations are either immediately observed, sealing the cover glass with paraffin or other means, or mounted permanently in a suitable resin. The Feulgen reaction only reveals the thymonucleotides, while other nucleal components of proteic, ribonucleic and lipidic nature are not colored. Despite the fact that some combinations with other dyes, for instance with light green, are also used after the Feulgen reaction, the lack of good morphological colorations of the cell elements is felt on many occasions when studying structures without thymonucleic acid.

It should be important to have a procedure equivalent to the nucleal squash method for the rapid mounting of materials colored in hematoxylin or coal tar dyes, etc. Such a method is here described, which we have used for some time in cytological work with good results.

### THE GLYCERIN SQUASH TECHNIC

The method consists in staining, after a suitable fixation, small pieces (thickness about 0.5–1 mm.) or freehand sections of the tissues in the desired dye, washing thoroughly, squashing or smearing in glycerin, and mounting in resin. The steps of the procedure are the following:

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1. Immerse the colored materials in pure glycerin for about 5 minutes to 2 hours, according to the thickness and the hardness of the pieces, for penetration. If the materials are liable to shrinkage in pure glycerin, use glycerin and distilled water (1:1) or glycerin and 70% alcohol (1:1). Sometimes the glycerin-water (1:1) gives better penetration than pure glycerin.
2. Squash the material by placing it in a drop of pure glycerin on a slide and pressing rapidly but gently between slide and cover glass. Rapid alternating strokes (allowing the cover slip to rise between strokes) give a good separation of the cells.
3. The preparation may be observed in glycerin after sealing with a cement made of lanolin and rosin (2 to 8 parts in weight). Permanent preparations may be easily obtained by mounting in rosin according to steps 4-7.
4. To mount permanently: Immerse the preparation overnight in distilled water in a jar, to about  $\frac{1}{3}$  to  $\frac{1}{2}$  of the cover glass height.
5. After about 12 hours in distilled water immerse the preparation with the cover glass in position (we have never observed a spontaneous detachment of the cover glass in water) in a jar with 96% alcohol. Here also, the level of the alcohol must be about  $\frac{1}{3}$  to  $\frac{1}{2}$  of the cover glass height.
6. About 12-24 or more hours after immersion in alcohol, the cover glass detaches spontaneously, or preferably, is lifted up with the aid of a fine-tipped lancet needle. Usually no loss of material is experienced in this step, the glycerin causing in general a better adherence than acetic acid in the nucleal method.
7. Rinse carefully in 96% alcohol for 3-5 minutes, maintaining the slide and cover glass horizontally, and mount in Euparal or other alcohol-soluble resins.

#### NOTES

If it is desired to mount in xylene soluble resins, it is necessary, of course, to pass through absolute alcohol and xylene, after step 6. It is better, however, to use alcohol-soluble resins, because in successive passages the materials are subject to loss and in many cases the contraction accompanying the treatments with absolute alcohol and xylene considerably diminishes the volume of structures already in the limit of visibility.

The method is applicable to all stains which do not deteriorate in a rapid passage through glycerin. Acetic acid can not be employed

instead of glycerin owing to its action upon hematoxylin and coal tar dyes.

The glycerin squash method can also be applied after a Feulgen reaction, alone or combined with an aqueous counterstaining. It may also be combined with stains dissolved in 96% alcohol, if the stain is applied when step 7 is reached.

The principal advantages of the method are: the saving of time in embedding and sectioning; the possibility of obtaining complete cells not cut by sectioning; and the use of almost any stain before squashing.



## LABORATORY HINTS FROM THE LITERATURE

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A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS  
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

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### MICROSCOPE AND OTHER APPARATUS

SALMON, M. V. **Practical phase-contrast microscopy.** *The Microscope*, 6, 177-88. 1947.

The author refers to recent interest in the phase-contrast microscope and explains fairly simply the principles underlying this method of making invisible differences of light phase visible by converting them into visible differences in intensity. He states that the only bar to widespread use of the method has been the difficulty in obtaining the phase discs for use in the objective. The writer describes a method of preparing such a disc, with an etched stripe half a wavelength deep, and of inserting it in an objective. He describes how this should be used in conjunction with the proper slit in the illuminant. Photomicrographs, showing detail brought out by phase-contrast but invisible by ordinary illumination, are given. The paper is of value to microscopists with mechanical ingenuity who are prepared to make such equipment for themselves, rather than to those who depend on manufacturers for all such specialized items.—*H. J. Conn.*

### MICROTECHNIC IN GENERAL

EVANS, TITUS C. **Radioautographs in which the tissue is mounted directly on the photographic plate.** *Proc. Soc. Exp. Biol. and Med.*, 64, 813-15. 1947.

Sections of tissue which contain radioactive material are mounted directly on the emulsion of a photographic plate in the dark room. Paraffin sections are floated on warm water in the usual manner to expand them and then transferred to cool water. The photographic plate, emulsion side up, is slipped under the section. One corner of the section is held against the emulsion while the plate plus the tissue are removed from the water. The preparation is then dried before a fan and placed in a light-tight box. The time required for the radiation period is not stated and it seems likely that it would have to be determined for the type of experimental procedure in use. After radiation, the paraffin is removed from the section with xylene, the preparation dried, and the photographic image developed with Eastman D-11 or D-72 solution. Fixation in acid hypo, washing and drying follow with subsequent staining by hematoxylin and eosin. If it is desirable to use photographic film instead of plates, several methods are suggested for avoiding overstaining of the film. The article is illustrated by three figures.—*H. A. Davenport.*

### DYES AND THEIR BIOLOGICAL USES

TOLBERT, B. M., and BRANCH, G. R. K. **The spectra of the doubly charged positive ions of some p,p'-diaminotriphenylmethane dyes.** *J. Amer. Chem. Soc.*, 69, 1083-91. 1947.

Spectra of the second ions of malachite green and some more highly phenylated diaminotriphenylmethane dyes were measured. Phenylation has a much greater bathochromic effect on the x-band of a second ion than on the corresponding band of a first ion, and the frequency differences between the x-bands and y-bands are greater in second ions than in first ions. Spectra of solutions containing both first and second ions are not linear combinations of the spectra of the first and second ions.—*R. T. Whittenberger.*

VAN WYK, J. J., and CLARK, W. M. The luminosity and chromaticity of indicators as a function of pH. *J. Amer. Chem. Soc.*, 69, 1296-1301. 1947.

The "one-color" indicator, p-nitrophenol, has a dominant wave-length of 568 m $\mu$  independent of pH. At the concentration used the indicator exhibits only about 2% change of luminosity between pH 6.1 and 7.7 but a four-fold change of spectral purity. Thus, discrimination between solutions of different pH is based almost exclusively on the change of spectral purity. In contrast, phenol red and brom thymol blue show distinct changes in luminosity, dominant wave-length and purity. The present state of analysis of factors concerned in the discrimination between small color differences does not permit the weighting of the effects of changes in luminosity and changes of chromaticity. However, certain minimal differences in chromaticity are indicated.—*R. T. Whittenberger.*

### ANIMAL MICROTCHNIC

HAMRE, CHRISTOPHER J. Hematopoiesis in the bone marrow of rats recovering from nutritional anemia. *J. Lab. & Clin. Med.*, 32, 756-76. 1947.

A study of blood and bone marrow was made in 60 anemic rats during the initial 48 hours of recovery after treatment with copper and iron. Hemoglobin and erythrocytes began to increase within 26 hours. Normoblasts, eosinophiles and heterophiles increased at 12 hours and continued high up to 26 to 28 hours. The first histologic response in the bone marrow was the development of heteroplastic erythropoietic tissue, including numerous pronormoblasts. This was greatest at 18 to 26 hours. At the same period there was great increase in normoblasts, eosinophiles and heterophiles, and these were discharged into the blood stream. This was followed by a period of homoplastic development. There was no rupture or destruction of the lining membrane of the sinuses during discharge of cells from the sinusoidal tissue into the sinuses. "Primitive cell" progenitors of granulocytes were not recognized in the preparations.—*John T. Myers.*

LIMARZI, LOUIS R. Evaluation of bone marrow concentration techniques. A modified method for the simultaneous preparation and staining of blood and bone marrow films. *J. Lab. & Clin. Med.*, 32, 732-40. 1947.

The author proposes the following technic: Under local procaine anaesthesia, introduce a 16 gauge sternal puncture needle at the second interspace, remove exactly 1.0 ml. of sternal fluid and place it in a paraffin-lined tube containing a minute amount of heparin. Mix, transfer to a Wintrobe hematocrit tube and centrifuge 5 minutes at 2000 r.p.m. Remove the fat and most of the plasma. Place one drop of the concentrated cells on a slide, draw a second slide at an angle of 30 to 45° against the drop until it spreads out in the angle, drop the top slide to "sandwich" the drop and remove the top slide. At the opposite end of the slide prepare a similar film with blood from finger or ear. Air dry and stain with Wright's stain. This method avoids dilution of the marrow fluid with peripheral blood and is simple and accurate.—*John T. Myers.*

HEATH, O. V. S. Role of starch in light-induced stomatal movement, and a new reagent for staining stomatal starch. *Nature*, 159, 647-8. 1947.

A reagent which satisfactorily stains leaf starch without causing stomatal closure is prepared as follows: dissolve 2.5 g. of iodine in 100 ml. of phenol, containing the minimum amount of water to make it liquid at room temperature and an excess of KI. This reagent has a very high refractive index, and excellent images may be obtained, either for visual work or photomicrography, by mounting the material in it.—*C. Randall.*

### MICROÖRGANISMS

COHEN, H. A. A new quick method for staining *Treponema pallidum*. *Acta Med. Orientalia*, 6, 99-100. 1947.

This staining method, which takes but 1 min., is based chemically on the reduction of AgNO<sub>3</sub> in an alkaline solution. The technic described is the following: (1) A thin film is essential and has, moreover, to be fixed immediately; (2)

Fixation is accomplished by a 36–40% formalin solution with subsequent heating until evaporation occurs. This is followed by flushing with distilled water; (3) Staining: 4 drops of a solution consisting of equal parts of  $N/1$  NaOH and  $N/10$  NaOH, are added followed by 2 drops of a 5%  $FeCl_3$  solution, 4 drops of  $N/1$   $NH_4OH \cdot HCl$  (hydroxylamine hydrochloride) and 3 drops of a 10% solution of pyrogallal acid. The slide is then heated until bubbles appear under simultaneous gentle stirring. This is followed by flushing with distilled water, after which a few drops of a 0.5–5% ammoniacal solution of  $AgNO_3$  are added. The slide is then washed and dried. After staining, according to this method, *Treponema* cells assume a reddish-brown color.—*Jas.* (Courtesy *Biological Abstracts*).

HENRY, H., and STACEY, M. **Histochemistry of the Gram-staining reaction for micro-organisms.** *Proc. Roy. Soc., Ser. B. Biol. Sci.*, 133, 391–406. 1946.

Extraction of *Clostridium welchii* cells with 2% bile salt in normal saline at 60°C. for 12 to 144 hr. extracts one-fourth of the dry weight, leaving a residue of Gram-negative cytoskeletons containing large amounts of a basic protein. Magnesium ribonucleate, isolated from the bile salt extract or prepared from commercial yeast nucleic acid, could be recombined in from 20 min. to several hours with Gram-negative cytoskeletons that were rendered receptive by 18 to 48 hr. treatment with 1% solution of reducing agents such as  $Na_2SO_3$ , glyoxal,  $NaHSO_3$ , ascorbic acid, dihydroxymaleic acid, thioglycollic acid or formaldehyde. Following recombination with magnesium ribonucleate, the cytoskeletons regained their Gram-positive reaction. Gram-stainable material in normal and reconstituted cells appears as opaque granules in ultraviolet photomicrographs (265  $m\mu$ ) corresponding to the strong absorption band of magnesium ribonucleate at 260  $m\mu$ . Bile salt extraction of *Saccharomyces cerevisiae* removed one-tenth of the dry weight, producing Gram-negative cytoskeletons which could be reconverted to Gram-positive by reduction and treatment with magnesium ribonucleate prepared from either yeast, *Clostridium welchii*, or *Bacillus subtilis*. Similar results obtained with *B. oedematiens*, *B. sporogenes*, *Vibrio septicum*, *B. anthracis*, *B. mycoides*, *B. mesentericum*, *B. megatherium*, and certain strains of streptococci and sarcinae are not reported in detail.—*E. H. Shaw, Jr.* (Courtesy *Biological Abstracts*).

PARA, M. **Silver impregnation of spirochetes in tissue sections.** *Arch. Path.*, 42, 649. 1946.

A new silver impregnation technic is described, which is applied in two possible procedures, the basic and rapid methods. Tissues fixed in a 1:10 dilution in saline of U.S.P. formaldehyde and paraffin sections 4–6  $\mu$  thick are used.

The basic procedure is as follows: Immerse deparaffined and hydrated sections 30 min. at room temperature in 1%  $UO_2(NO_3)_2 \cdot 6H_2O$ , (or 45 min. at 56°C. in 4% aqueous ferrous ammonium sulfate, or 10–15 min. in a sulfur water mixture made up by adding 10 ml. 5% anhydrous  $NaHSO_3$  and 10 ml.  $N$  HCl to 200 ml. distilled water in a tightly stoppered bottle, or 20 min. at 56°C. in 1% oxalic acid solution, or 30 min. at room temperature in 0.5%  $CuSO_4$  or 5 min. at room temperature in 0.02%  $K_2MnO_4$ ). Wash rapidly in distilled water. Treat 2 hr. at 56°C. with 1.5% aqueous  $AgNO_3$  (stored in the dark), replacing the silver solution after the first hour with fresh solution, or overnight at 37°C. with the same silver solution. Wash rapidly in distilled water. Treat 1 hr. at room temperature with the following, made up just before use: 5 ml. 5% rosin (colophony) in ethanol are added with gentle shaking to 45 ml. of a solution prepared by adding 2.0 ml. saturated aqueous  $Li_2CO_3$  (about 1.3%) to 100 ml. boiling 0.2% aqueous  $AgNO_3$ , boiling 1–2 minutes and filtering through paper (this solution will keep one month in the refrigerator), (or 1 hr. at room temperature with colloidal silver tartrate solution made up by adding 10 ml. 5% alcoholic rosin to 40 ml. of Steiner's double tartrate-silver-nitrate solution, or 1 hr. at room temperature in a mixture of 10 ml. of 5% alcoholic rosin and 40 ml. of 10% aqueous dilution of ox bile, followed by 2 hr. at 56°C. in 1.5% aqueous  $AgNO_3$ ). Immerse 10–15 min. at room temperature in Levaditi's reducing solution consisting of 4% aqueous pyrogallal acid with 5% by volume of USP formalin. Wash rapidly in distilled water. (If microscopic examination at this time shows improper tissue impregnation, treat section rapidly with absolute ethanol, im-

merse 10 min. in the  $\text{AgNO}_3$ -rosin mixture and 10 min. in Levaditi's reducing solution.) Dehydrate, and mount in Canada balsam.

Sections impregnated with the above technic are yellow-brown in color and show brown cell nuclei and black spirochaetes. If the tissue is not properly fixed a poor silver impregnation results. In such a case, cut new paraffin sections from the block; mount, remove paraffin and hydrate to the 50% ethanol stage; treat 1 hr. at room temperature with 2% alcoholic rosin solution; wash in absolute ethanol; rehydrate; and impregnate as previously described.

The following substances may be substituted for the rosin in the sensitizing mixture: gum mastic (add 10 ml. of 2.5% absolute ethanolic gum mastic solution to 40 ml. of  $\text{AgNO}_3\text{-Li}_2\text{CO}_3$  solution or Steiner's silver tartrate solution); ox bile (add 5 ml. of fresh ox bile to 4.5 ml. of  $\text{AgNO}_3\text{-Li}_2\text{CO}_3$  solution or Steiner's silver tartrate solution); balsam of tolu (add 5 ml. 1% ethanolic balsam of tolu solution to 45 ml. of  $\text{AgNO}_3\text{-Li}_2\text{CO}_3$  solution). A solution of 1 g. hydroquinone in 60 ml. distilled water may be substituted for the pyrogallol-acid-formaldehyde reducing solution.

The procedure for the rapid method is as follows: Hydrate sections after removal of paraffin; treat 1 hr. at  $56^\circ\text{C}$ . with a freshly mixed preparation containing 10 ml. of 1% aqueous  $\text{AgNO}_3$ , 10 ml. of a dilution of 1 ml. of 1.3%  $\text{Li}_2\text{CO}_3$  in 165 ml. of distilled water (or 10 ml. of 0.1% sodium potassium tartrate solution) and 10 ml. of 2% ethanolic rosin solution (dilute filtered 50% rosin 25 times with absolute ethanol just before use); treat 7-10 min. at room temperature in a reducing mixture consisting of 50 ml. of Levaditi's reducing solution and 5 ml. of 2% ethanolic rosin solution; wash 5 min. in each of 2 or 3 baths of 90% ethanol; wash rapidly in distilled water; replace 15 min. at  $56^\circ\text{C}$ . in the  $\text{AgNO}_3\text{-Li}_2\text{CO}_3$ -rosin mixture; treat 3-5 min. in the above reducing mixture; wash 2 min. in each of 2 baths of 90% ethanol; dehydrate and mount in Canada balsam.

A combined process for studying pathological changes in the preparations as well as demonstrating spirochaetes is also described. This is as follows: Take tissue section through the rapid impregnation procedure up to the dehydration step, except that no rosin is used in the reducing solution; wash in distilled water; stain 10-15 min. in Harris' hematoxylin; pass rapidly through the dilution of 1 ml. 1.3%  $\text{Li}_2\text{CO}_3$  in 165 ml. distilled water; wash in running water; stain 1-3 min. in Altmann's acid fuchsin diluted 5 times in distilled water before use; differentiate in absolute ethanol for 5 min.; dehydrate and mount in Canada balsam.

With the above technic the cytoplasm is stained light red, the nuclei dark violet, the connective tissues bright red or ochre yellow and the spirochaetes black.

Von Gieson's stain for 1 min. may be used in place of Altmann's acid fuchsin. Meyrick and Harrison's stain (15 vol. 1% aqueous neutral red and 1 vol. carbol fuchsin made by adding 9 ml. 10% alcoholic basic fuchsin to 90 ml. 5% aqueous phenol) for 10 min. may be substituted for the hematoxylin stain followed by rapid differentiation in absolute ethanol, dehydration and mounting in Canada balsam. With this latter staining modification the cytoplasm is stained light red, the nuclei bright red, the connective tissues dark red or greenish yellow and the spirochaetes black.—*L. Farber*.

**RUIZ, MERINO, J.** A method of staining capsules. *Rev. Sanidad e Hig. Publ.* (Madrid), 20, 1112. 1946.

This easy method requires the bacteria to be growing under conditions to obtain the largest development of their capsules. The reagents used are: Solution A, methyl violet, 1%; Solution B, yellowish eosin, 0.01 g., distilled water, 10 ml., horse normal serum, 3 ml.

One drop of reagent A is mixed with the material to be stained using a platinum needle and left 5 min. in a moist room to avoid evaporation; then one drop of solution B is added, mixing it for 3 min., and with a glass slip a smear like those of hematology is made; it is observed with a microscope after drying.—*I. Pemandar*. (Courtesy *Biological Abstracts*).

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